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(54) Title: ALPHAVIRUS VECTORS AND VIROSOMES WITH MODIFIED HIV GENES FOR USE AS VACCINES

(57) Abstract: The present invention provides methods and compositions comprising a population of alphavirus replicon particles comprising two or more isolated nucleic acids selected from 1) an isolated nucleic acid encoding an *env* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, 2) an isolated nucleic acid encoding a *gag* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, wherein the *gag* gene product or immunogenic fragment thereof is modified to inhibit formation of virus-like particles containing the *gag* gene product or the immunogenic fragment thereof and their release from a cell, and 3) an isolated nucleic acid encoding a *pol* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, wherein the *pol* gene product or immunogenic fragment thereof is modified to inhibit integrase, RNase H and/or reverse transcriptase activity, and wherein the nucleic acids are each contained within a separate alphavirus replicon particle.

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**ALPHAVIRUS VECTORS AND VIROSOMES
WITH MODIFIED HIV GENES FOR USE AS VACCINES**

This application claims priority to U.S. Provisional Application No. 60/216,995,
5 filed July 7, 2000 which is hereby incorporated by reference in its entirety.

BACKGROUND OF THE INVENTION

Field of the Invention

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The present invention relates to vaccines using viral antigens, and in particular, to vaccines for the treatment and prevention of human immunodeficiency virus (HIV) infection. The vaccines of this invention comprise alphavirus RNA replicon systems which contain nucleic acid sequence encoding antigens for eliciting an immune 15 response to HIV.

Background

The successful control of the AIDS epidemic will require an effective vaccine 20 for human immunodeficiency virus type 1 (HIV) that significantly reduces or prevents the spread of infection. Currently, several viral vector systems as well as naked DNA are at various stages of pre-clinical and clinical evaluation as candidate HIV vaccines. Recombinant poxviruses are the most widely studied virus vectors and are furthest along in clinical development (e.g., ALVAC).

25

The alphavirus-based replicon particle systems, such as the ones described in U.S. Patent No. 5,792,462 and herein referred to as "VRPs," have multiple distinct properties that make them attractive as an HIV vaccine delivery technology. These properties include: natural targeting to and expression in lymphoid tissues (an optimal 30 site for induction of an immune response); high antigen expression levels, e.g., up to 20% of total cell protein; induction of balanced humoral, cellular, and mucosal immune responses; sustained efficacy over multiple simultaneous or sequential inoculations of

the vector; and a high margin of safety.

Venezuelan equine encephalitis virus (VEE) is a member of the Alphaviruses group, which also includes the prototype Sindbis virus (SIN) and Semliki Forest virus

5 (SFV), and is comprised of enveloped viruses containing plus-stranded RNA genomes within icosahedral capsids (Strauss, 1994). Alphavirus genomes are: approximately 11.5 kb long, capped, polyadenylated, and infectious under appropriate transfection conditions. The nucleocapsid is composed of 240 molecules of the capsid protein arranged as a T=4 icosahedron, and is surrounded by a lipoprotein envelope (Paredes *et al.*, 1993). Protruding from the virion surface are 80 glycoprotein spikes, each of which is a trimer of virally encoded E1 and E2 glycoprotein heterodimers. The virions contain 10 no host proteins.

Alphaviruses share replication strategies and genomic organization. The 15 complete replicative cycle of alphaviruses occurs in the cytoplasm of infected cells. Expression from the alphavirus genome is segregated into two regions. The four enzymatic nonstructural proteins (nsP1-nsP4) are synthesized from the 5' two-thirds of the genome-length RNA and are required for RNA replication. Immediately following 20 infection, the nsPs are produced by translation of parental genomes and catalyze the synthesis of a full-length negative-sense copy of the genome. This serves as a template for the synthesis of progeny plus-stranded genomes.

The negative-sense copy of the genome also serves as the template for the 25 synthesis of subgenomic mRNA at approximately 10-fold molar excess relative to genomic RNA in infected cells (Schlesinger and Schlesinger, 1990). Synthesis of subgenomic 26S mRNA is initiated from the highly active internal 26S mRNA promoter, which is functional only on the negative-sense RNA. The subgenomic mRNA corresponds to the 3' one-third of the genome and encodes the alphavirus structural proteins.

Full-length, infectious cDNA clones of the RNA genome of VEE (Davis *et al.*, 1989) have been constructed, a panel of mutations which strongly attenuate the virus have been identified (Johnston and Smith, 1988; Davis *et al.*, 1990), and various constellations of these attenuating mutations have been inserted into the clones to 5 generate several live attenuated VEE vaccine candidates (Davis *et al.*, 1991; 1995b; Grieder *et al.*, 1995). The resulting vaccine candidates are avirulent and provide complete protection against lethal virus challenge in rodents, horses and nonhuman primates.

10 The alphavirus VRPs are propagation defective, single cycle vectors that contain a self-amplifying alphavirus RNA (replicon RNA) in which the structural protein genes of the virus are replaced by a heterologous antigen gene to be expressed. Alphavirus VRPs are typically made in cultured cells, referred to as packaging cells. Following introduction into mammalian cells, the replicon RNA is packaged into VRP 15 by supplying the structural proteins in "trans," i.e. the cells are co-transfected with both replicon RNA and one or more separate helper RNAs which together encode the full complement of alphavirus structural proteins. Importantly, only the replicon RNA is packaged into VRP, as the helper RNA(s) lack the *cis*-acting packaging sequence required for encapsidation. Thus, the VRPs are defective, in that they can only infect 20 target cells in culture or *in vivo*, where they express the heterologous antigen gene to high level, but they lack critical portions of the VEE genome (i.e., the VEE structural protein genes) necessary to produce virus particles which could spread to other cells.

Delivery of the replicon RNA into target cells (for vaccination) is facilitated by 25 the VRP following infection of the target cells. In the cytoplasm of the target cell, the replicon RNA is first translated to produce the viral replicase proteins necessary to initiate self-amplification and expression. The heterologous antigen gene is encoded by a subgenomic mRNA, abundantly transcribed from the replicon RNA, leading to high level expression of the heterologous antigen gene product. Since the VEE structural 30 protein genes are not encoded by the replicon RNA delivered to the target cell, progeny

virion particles are not assembled, thus limiting the replication to a single cycle within the infected target cell. Experimental VRP vaccines have been successful in vaccinating rodents against influenza virus, Lassa fever virus and Marburg virus (Pushko *et al.*, 1997; Hevey *et al.*, 1998). In nonhuman primates, VRP vaccines have 5 demonstrated complete efficacy against lethal Marburg virus challenge (Hevey *et al.*, 1998), shown partial but significant protection against SIV infection and disease (Davis *et al.*, 2000) and induced an anti-HA response at a level consistent with protection of humans against influenza virus infection.

10 The alphavirus based replicon vector systems, and in particular the VEE-based systems, present several advantages in vaccination, including safety and high immunogenicity/efficacy. VEE is unique among the alphaviruses in that a live attenuated IND VEE vaccine, TC-83, (Kinney *et al.*, 1989; Kinney *et al.*, 1993) has been inoculated into approximately 8,000 humans. This allows direct safety and 15 efficacy comparisons between human, nonhuman primate and rodent responses to the same VEE derivative. A large body of experience strongly suggests that the animal models generally reflect the human susceptibility and disease course, except that mice are far more susceptible to lethal VEE disease than humans or nonhuman primates. Furthermore, the VEE replicon vectors express high levels of the gene of interest in cell 20 culture, and *in vivo* expression is targeted to lymphoid tissues, reflecting the natural tropism mediated by the VEE glycoproteins. Cells in the draining lymph node of VRP-inoculated mice contain detectable amounts of the desired gene product within hours of inoculation. This expression continues for up to five days.

25 To date, VRP vector vaccines have been used in over 2000 rodents and in 94 macaques at doses up to 5×10^8 i.u., with no indication of any clinical manifestations.

30 In work reported by Pushko *et al.* (1997), individual mice were immunized sequentially with Lassa virus N-VRP and influenza virus HA-VRP. Groups of mice, which received two inoculations of 3×10^4 or 3×10^6 i.u. of Lassa N-VRP followed by

two inoculations of 2×10^5 i.u. of HA-VRP, all responded with serum antibodies to both antigens. The level of anti-influenza antibody induced in these sequentially inoculated mice was equivalent to a control group, which received two inoculations of buffer followed by two inoculations of 2×10^5 i.u. of HA-VRP. All HA-VRP 5 immunized mice were completely protected against influenza virus challenge. Furthermore, sequential immunization of mice with two inoculations of N-VRP prior to two inoculations of HA-VRP induced an immune response to both HA and N equivalent to immunization with either VRP construct alone. Primary and booster immunization with a VRP preparation expressing an immunogen from one pathogen 10 did not interfere with the development of a protective response to subsequent primary immunization and boosting with VRP expressing an immunogen from a second pathogen, thus showing that the VRP-based system can be used to induce immunity to a variety of pathogens in the same individual over time.

15 Four macaques were inoculated subcutaneously at week 0 with 10^5 i.u. each of SIV-gp160-VRP (*env*) and SIV MA/CA-VRP (*gag*), boosted by the same route at week 7 with 10^7 i.u. of each VRP vaccine, and intravenously at weeks 12 and 20 with 5×10^8 i.u. of each VRP. Two control animals were inoculated with equivalent doses of HA-VRP (haemagglutinin, a glycoprotein from influenza virus), and two with the vehicle 20 only. The four SIV-VRPs immunized monkeys received subcutaneously an additional dose of 2×10^7 i.u. of gp140-VRP at week 41, followed by a final boost of 2×10^7 i.u. each of gp140-VRP and MA/CA-VRP at week 49. Four weeks after the final immunization, all eight macaques were challenged intravenously with the pathogenic virus, SIVsmE660.

25

After these inoculations, three of four test macaques had measurable CTL-specific killing directed against both SIV *gag* and *env*, all four had gp160 IgG antibody by ELISA, and the three animals which harbored SIV-specific CTL also showed neutralizing antibody to SIVsmH-4.

Four of four vaccinated animals were protected against disease for at least 16 months following intravenous challenge with the pathogenic SIV swarm, while the two vehicle controls required euthanasia at week 10 and week 11, post challenge. In two of the vaccinees, plasma virus levels were below the limit of detection by branched chain 5 DNA assay. At 64 weeks post challenge, all four vaccinated animals showed no clinical signs of disease. One animal remained vDNA negative at 64 weeks.

The results of this highly pathogenic challenge demonstrated that the immune response induced by vaccination with SIV-VRP was effective in preventing early 10 mortality and increasing the ability to suppress challenge virus replication. The ability to control SIV replication and reduce viral load to undetectable levels was closely correlated with the strongest measurable antibody and cellular immune responses.

While these results are encouraging, the level of protection obtained would not 15 be acceptable for a human vaccine against HIV infection. Thus, there remains a need for a robust, effective and safe vaccine against HIV infection in humans. Development of a HIV vaccine comprising the complete, or immunogenic fragments of the, *gag* gene (Gag-VRP), an immunogenic portion of the *pol* gene (Pol-VRP), and the complete, or immunogenic fragments of the, *env* gene (Env-VRP), would increase the diversity of 20 available CTL epitopes substantially and thus address this need.

SUMMARY OF THE INVENTION

The present invention provides a composition comprising two or more isolated 25 nucleic acids selected from the group consisting of an isolated nucleic acid encoding an *env* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, an isolated nucleic acid encoding a *gag* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, wherein the *gag* gene product or immunogenic fragment thereof is modified to inhibit formation of virus-like particles 30 containing the *gag* gene product or the immunogenic fragment thereof and their release

a cell, and an isolated nucleic acid encoding a *pol* gene product or an unogenic fragment thereof of a human immunodeficiency virus, wherein the *pol* product or immunogenic fragment thereof is modified to inhibit reverse transcriptase activity.

Also provided is a composition comprising a population of alphavirus replicon particles comprising two or more isolated nucleic acids selected from the group consisting of 1) an isolated nucleic acid encoding an *env* gene product or an unogenic fragment thereof of a human immunodeficiency virus, 2) an isolated nucleic acid encoding a *gag* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, wherein the *gag* gene product or immunogenic fragment thereof is modified to inhibit formation of virus-like particles containing the *gag* gene product or the immunogenic fragment thereof and their release from a cell, 3) an isolated nucleic acid encoding a *pol* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, wherein the *pol* gene product or unogenic fragment thereof is modified to inhibit reverse transcriptase activity, and wherein the nucleic acids are each contained within a separate alphavirus replicon particle.

In addition, the present invention provides a composition comprising a population of alphavirus replicon particles comprising two or more isolated nucleic acids selected from the group consisting of 1) an isolated nucleic acid encoding an *env* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, 2) an isolated nucleic acid encoding a *gag* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, wherein the *gag* gene product or immunogenic fragment thereof is modified to inhibit formation of virus-like particles containing the *gag* gene product or the immunogenic fragment thereof and their release from a cell, and 3) an isolated nucleic acid encoding a *pol* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, wherein the *pol* gene product or immunogenic fragment thereof is modified to inhibit reverse transcriptase activity.

transcriptase activity, and wherein the nucleic acids are each contained within a separate alphavirus replicon particle, and further wherein the alphavirus replicon particles comprise a replicon RNA or at least one structural protein which comprises one or more attenuating mutations.

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10. A method of making a population of alphavirus replicon particles of this invention is provided herein, comprising:

A) (a) providing a first helper cell for producing a first population of infectious, replication defective alphavirus particles, comprising in an alphavirus-permissive cell:

10 (i) an alphavirus replicon RNA, wherein the replicon RNA comprises an alphavirus packaging signal and a nucleic acid encoding an *env* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, and wherein the replicon RNA lacks sequences encoding alphavirus structural proteins;

15 (ii) a first helper RNA separate from said replicon RNA, said first helper RNA encoding at least one alphavirus structural protein and furthermore not encoding at least one other alphavirus structural protein; and

20 (iii) one or more additional helper RNA(s) separate from said replicon RNA and separate from said first helper RNA, said additional helper RNA(s) encoding at least one other alphavirus structural protein not encoded by said first helper RNA;

and with at least one of said helper RNAs lacking an alphavirus packaging signal;

25 wherein the combined expression of the alphavirus replicon RNA and the helper RNAs produces an assembled alphavirus particle which is able to infect a cell, and is unable to complete viral replication, and further wherein the first population contains no detectable replication-competent alphavirus particles as determined by passage on permissive cells in culture;

30 (b) producing the alphavirus particles in the helper cell; and

(c) collecting the alphavirus particles from the helper cells;

B) (a) providing a second helper cell for producing a second population of infectious, replication defective alphavirus particles, comprising in an alphavirus-
5 permissive cell:

(i) an alphavirus replicon RNA, wherein the replicon RNA comprises an alphavirus packaging signal and a nucleic acid encoding a *gag* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, wherein the *gag* gene product or immunogenic fragment thereof is modified to inhibit formation of virus-like particles containing the *gag* gene product or the immunogenic fragment thereof and their release from a cell, and wherein the replicon RNA lacks sequences encoding alphavirus structural proteins;

10 (ii) a first helper RNA separate from said replicon RNA, said first helper RNA encoding at least one alphavirus structural protein and furthermore not encoding at least one other alphavirus structural protein; and

15 (iii) one or more additional helper RNA(s) separate from said replicon RNA and separate from said first helper RNA, said additional helper RNA(s) encoding at least one other alphavirus structural protein not encoded by said first helper RNA;

20 and with at least one of said helper RNAs lacking an alphavirus packaging signal;

25 wherein the combined expression of the alphavirus replicon RNA and the helper RNAs produces an assembled alphavirus particle which is able to infect a cell, and is unable to complete viral replication, and further wherein the second population contains no detectable replication-competent alphavirus particles as determined by passage on permissive cells in culture;

(b) producing the alphavirus particles in the helper cell; and

30 (c) collecting the alphavirus particles from the helper cells;

C) (a) providing a third helper cell for producing a third population of infectious, replication defective alphavirus particles, comprising in an alphavirus-permissive cell:

5 (i) an alphavirus replicon RNA, wherein the replicon RNA comprises an alphavirus packaging signal and a nucleic acid encoding a *pol* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, wherein the *pol* gene product or immunogenic fragment thereof is modified to inhibit reverse transcriptase activity, and wherein the replicon RNA lacks sequences encoding alphavirus structural proteins;

10 (ii) a first helper RNA separate from said replicon RNA, said first helper RNA encoding at least one alphavirus structural protein and furthermore not encoding at least one other alphavirus structural protein; and

15 (iii) one or more additional helper RNA(s) separate from said replicon RNA and separate from said first helper RNA, said additional helper RNA(s) encoding at least one other alphavirus structural protein not encoded by said first helper RNA;

20 and with at least one of said helper RNAs lacking an alphavirus packaging signal;

wherein the combined expression of the alphavirus replicon RNA and the helper RNAs produces an assembled alphavirus particle which is able to infect a cell, and is unable to complete viral replication, and further wherein the third population contains no detectable replication-competent alphavirus particles as determined by passage on

25 permissive cells in culture;

(b) producing the alphavirus particles in the helper cell; and

(c) collecting the alphavirus particles from the helper cells; and

D) combining the first population of alphavirus particles produced from the first helper cell, the second population of alphavirus particles produced from the second

helper cell and the third population of alphavirus particles produced from the third helper cell, thereby producing the population of alphavirus replicon particles.

Also provided is a method of making a population of alphavirus replicon
5 particles, comprising:

A) (a) providing a first helper cell for producing a first population of infectious, replication defective alphavirus particles, comprising in an alphavirus-permissive cell:

(i) an alphavirus replicon RNA, wherein the replicon RNA
comprises an alphavirus packaging signal and a nucleic acid encoding
10 an *env* gene product or an immunogenic fragment thereof of a human
immunodeficiency virus, and wherein the replicon RNA lacks sequences
encoding alphavirus structural proteins;

(ii) a first helper RNA separate from said replicon RNA, said first
helper RNA encoding at least one alphavirus structural protein and
15 furthermore not encoding at least one other alphavirus structural protein;
and

(iii) one or more additional helper RNA(s) separate from said
replicon RNA and separate from said first helper RNA, said additional
helper RNA(s) encoding at least one other alphavirus structural protein
20 not encoded by said first helper RNA;

and with at least one of said helper RNAs lacking an alphavirus packaging
signal;

wherein the combined expression of the alphavirus replicon RNA and the helper
RNAs produces an assembled alphavirus particle which is able to infect a cell, and is
25 unable to complete viral replication, and further wherein the first population contains
no detectable replication-competent alphavirus particles as determined by passage on
permissive cells in culture, and further wherein at least one of said replicon RNA, said
first helper RNA, and said one or more additional helper RNA(s) comprises one or
more attenuating mutations;

30 (b) producing the alphavirus particles in the helper cell; and

(c) collecting the alphavirus particles from the helper cells;

B) (a) providing a second helper cell for producing a second population of infectious, replication defective alphavirus particle, comprising in an alphavirus-5 permissive cell:

(i) an alphavirus replicon RNA, wherein the replicon RNA comprises an alphavirus packaging signal and a nucleic acid encoding a *gag* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, wherein the *gag* gene product or immunogenic fragment thereof is modified to inhibit formation of virus-like particles containing the *gag* gene product or the immunogenic fragment thereof and their release from a cell, and wherein the replicon RNA lacks sequences encoding alphavirus structural proteins;

(ii) a first helper RNA separate from said replicon RNA, said first helper RNA encoding at least one alphavirus structural protein and furthermore not encoding at least one other alphavirus structural protein; and

(iii) one or more additional helper RNA(s) separate from said replicon RNA and separate from said first helper RNA, said additional helper RNA(s) encoding at least one other alphavirus structural protein not encoded by said first helper RNA;

10 and with at least one of said helper RNAs lacking an alphavirus packaging signal;

15 wherein the combined expression of the alphavirus replicon RNA and the helper RNAs produces an assembled alphavirus particle which is able to infect a cell, and is unable to complete viral replication, and further wherein the second population contains no detectable replication-competent alphavirus particles as determined by passage on permissive cells in culture, and further wherein at least one of said replicon RNA, said first helper RNA, and said one or more additional helper RNA(s) comprises one or

20 more attenuating mutations;

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- (b) producing the alphavirus particles in the helper cell; and
- (c) collecting the alphavirus particles from the helper cells;

5 C) (a) providing a third helper cell for producing a third population of infectious, replication defective alphavirus particles, comprising in an alphavirus-permissive cell:

- (i) an alphavirus replicon RNA, wherein the replicon RNA comprises an alphavirus packaging signal and a nucleic acid encoding a *pol* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, wherein the *pol* gene product or immunogenic fragment thereof is modified to inhibit reverse transcriptase activity, and wherein the replicon RNA lacks sequences encoding alphavirus structural proteins;
- (ii) a first helper RNA separate from said replicon RNA, said first helper RNA encoding at least one alphavirus structural protein and furthermore not encoding at least one other alphavirus structural protein; and
- (iii) one or more additional helper RNA(s) separate from said replicon RNA and separate from said first helper RNA, said additional helper RNA(s) encoding at least one other alphavirus structural protein not encoded by said first helper RNA;

20 and with at least one of said helper RNAs lacking an alphavirus packaging signal;

25 wherein the combined expression of the alphavirus replicon RNA and the helper RNAs produces an assembled alphavirus particle which is able to infect a cell, and is unable to complete viral replication, and further wherein the third population contains no detectable replication-competent alphavirus particles as determined by passage on permissive cells in culture, and further wherein at least one of said replicon RNA, said first helper RNA, and said one or more additional helper RNA(s) comprises one or 30 more attenuating mutations;

- (b) producing the alphavirus particles in the helper cell; and
- (c) collecting the alphavirus particles from the helper cells; and

5 D) combining the first population of alphavirus particles produced from the first helper cell, the second population of alphavirus particles produced from the second helper cell and the third population of alphavirus particles produced from the third helper cell, thereby producing the population of alphavirus replicon particles.

Furthermore, the present invention provides a composition comprising two or 10 more isolated nucleic acids selected from the group consisting of an isolated nucleic acid encoding an *env* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, an isolated nucleic acid encoding a *gag* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, wherein the *gag* gene product or immunogenic fragment thereof is modified to inhibit formation of 15 virus-like particles containing the *gag* gene product or the immunogenic fragment thereof and their release from a cell, and an isolated nucleic acid encoding a *pol* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, wherein the *pol* gene product or immunogenic fragment thereof comprises a modification resulting in deletion or inactivation of integrase, RNase H and reverse 20 transcriptase functions in the *pol* gene product or immunogenic fragment thereof.

In addition, the present invention provides a composition comprising a population of alphavirus replicon particles comprising two or more isolated nucleic acids selected from the group consisting of 1) an isolated nucleic acid encoding an *env* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, 25 2) an isolated nucleic acid encoding a *gag* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, wherein the *gag* gene product or immunogenic fragment thereof is modified to inhibit formation of virus-like particles containing the *gag* gene product or the immunogenic fragment thereof and their release 30 from a cell, and 3) an isolated nucleic acid encoding a *pol* gene product or an

immunogenic fragment thereof of a human immunodeficiency virus, wherein the *pol* gene product or immunogenic fragment thereof comprises a modification resulting in deletion or inactivation of integrase, RNase H and reverse transcriptase functions in the *pol* gene product or immunogenic fragment thereof, and wherein the nucleic acids are
5 each contained within a separate alphavirus replicon particle.

Also provided herein is a composition comprising a population of alphavirus replicon particles comprising two or more isolated nucleic acids selected from the group consisting of 1) an isolated nucleic acid encoding an *env* gene product or an
10 immunogenic fragment thereof of a human immunodeficiency virus, 2) an isolated nucleic acid encoding a *gag* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, wherein the *gag* gene product or immunogenic fragment thereof is modified to inhibit formation of virus-like particles containing the *gag* gene product or the immunogenic fragment thereof and their release from a cell,
15 and 3) an isolated nucleic acid encoding a *pol* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, wherein the *pol* gene product or immunogenic fragment thereof comprises a modification resulting in deletion or inactivation of integrase, RNase H and reverse transcriptase functions in the *pol* gene product or immunogenic fragment thereof, and wherein the nucleic acids are each
20 contained within a separate alphavirus replicon particle, and further wherein the alphavirus replicon particles comprise a replicon RNA or at least one structural protein which comprises one or more attenuating mutations.

In these embodiments, the *gag* gene product or immunogenic fragment thereof
25 can be modified by mutation of the second codon, whereby a glycine is changed to an alanine and the *pol* gene product or immunogenic fragment thereof can be modified by mutation of the nucleotide sequence encoding the active site motif, whereby YMDD is changed to YMMA or HMMA. In addition, the *pol* gene product or immunogenic fragment thereof is modified to produce only p51 of the *pol* gene product or
30 immunogenic fragment thereof.

The present invention provides a method of making a population of alphavirus replicon particles, comprising:

A) (a) providing a first helper cell for producing a first population of infectious, replication defective alphavirus particles, comprising in an alphavirus-permissive cell:

5 (i) an alphavirus replicon RNA, wherein the replicon RNA comprises an alphavirus packaging signal and a nucleic acid encoding an *env* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, and wherein the replicon RNA lacks sequences encoding alphavirus structural proteins;

10 (ii) a first helper RNA separate from said replicon RNA, said first helper RNA encoding at least one alphavirus structural protein and furthermore not encoding at least one other alphavirus structural protein; and

15 (iii) one or more additional helper RNA(s) separate from said replicon RNA and separate from said first helper RNA, said additional helper RNA(s) encoding at least one other alphavirus structural protein not encoded by said first helper RNA;

and with at least one of said helper RNAs lacking an alphavirus packaging signal;

20 wherein the combined expression of the alphavirus replicon RNA and the helper RNAs produces an assembled alphavirus particle which is able to infect a cell, and is unable to complete viral replication, and further wherein the first population contains no detectable replication-competent alphavirus particles as determined by passage on permissive cells in culture;

25 (b) producing the alphavirus particles in the helper cell; and
(c) collecting the alphavirus particles from the helper cells;

B) (a) providing a second helper cell for producing a second population of infectious, replication defective alphavirus particles, comprising in an alphavirus-permissive cell:

(i) an alphavirus replicon RNA, wherein the replicon RNA comprises an alphavirus packaging signal and a nucleic acid encoding a *gag* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, wherein the *gag* gene product or immunogenic fragment thereof is modified to inhibit formation of virus-like particles containing the *gag* gene product or the immunogenic fragment thereof and their release from a cell, and wherein the replicon RNA lacks sequences encoding alphavirus structural proteins;

5 (ii) a first helper RNA separate from said replicon RNA, said first helper RNA encoding at least one alphavirus structural protein and furthermore not encoding at least one other alphavirus structural protein; and

10 (iii) one or more additional helper RNA(s) separate from said replicon RNA and separate from said first helper RNA, said additional helper RNA(s) encoding at least one other alphavirus structural protein not encoded by said first helper RNA;

15 and with at least one of said helper RNAs lacking an alphavirus packaging signal;

wherein the combined expression of the alphavirus replicon RNA and the helper

20 RNAs produces an assembled alphavirus particle which is able to infect a cell, and is unable to complete viral replication, and further wherein the second population contains no detectable replication-competent alphavirus particles as determined by passage on permissive cells in culture;

(b) producing the alphavirus particles in the helper cell; and

25 (c) collecting the alphavirus particles from the helper cells;

C) (a) providing a third helper cell for producing a third population of infectious, replication defective alphavirus particles, comprising in an alphavirus-permissive cell:

30 (i) an alphavirus replicon RNA, wherein the replicon RNA

comprises an alphavirus packaging signal and a nucleic acid encoding a *pol* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, wherein the *pol* gene product or immunogenic fragment thereof comprises a modification resulting in deletion or inactivation of integrase, RNase H and reverse transcriptase functions in the *pol* gene product or immunogenic fragment thereof, and wherein the replicon RNA lacks sequences encoding alphavirus structural proteins;

5 (ii) a first helper RNA separate from said replicon RNA, said first helper RNA encoding at least one alphavirus structural protein and furthermore not encoding at least one other alphavirus structural protein; and

10 (iii) one or more additional helper RNA(s) separate from said replicon RNA and separate from said first helper RNA, said additional helper RNA(s) encoding at least one other alphavirus structural protein not encoded by said first helper RNA;

15 and with at least one of said helper RNAs lacking an alphavirus packaging signal;

wherein the combined expression of the alphavirus replicon RNA and the helper RNAs produces an assembled alphavirus particle which is able to infect a cell, and is

20 unable to complete viral replication, and further wherein the third population contains no detectable replication-competent alphavirus particles as determined by passage on permissive cells in culture;

(b) producing the alphavirus particles in the helper cell; and

(c) collecting the alphavirus particles from the helper cells; and

25 D) combining the first population of alphavirus particles produced from the first helper cell, the second population of alphavirus particles produced from the second helper cell and the third population of alphavirus particles produced from the third helper cell, thereby producing the population of alphavirus replicon particles.

An additional method of making a population of alphavirus replicon particles is provided, comprising:

A) (a) providing a first helper cell for producing a first population of infectious, replication defective alphavirus particles, comprising in an alphavirus-permissive cell:

5 (i) an alphavirus replicon RNA, wherein the replicon RNA comprises an alphavirus packaging signal and a nucleic acid encoding an *env* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, and wherein the replicon RNA lacks sequences encoding alphavirus structural proteins;

10 (ii) a first helper RNA separate from said replicon RNA, said first helper RNA encoding at least one alphavirus structural protein and furthermore not encoding at least one other alphavirus structural protein; and

15 (iii) one or more additional helper RNA(s) separate from said replicon RNA and separate from said first helper RNA, said additional helper RNA(s) encoding at least one other alphavirus structural protein not encoded by said first helper RNA;

and with at least one of said helper RNAs lacking an alphavirus packaging signal;

20 wherein the combined expression of the alphavirus replicon RNA and the helper RNAs produces an assembled alphavirus particle which is able to infect a cell, and is unable to complete viral replication, and further wherein the first population contains no detectable replication-competent alphavirus particles as determined by passage on permissive cells in culture, and further wherein at least one of said replicon RNA, said 25 first helper RNA, and said one or more additional helper RNA(s) comprises one or more attenuating mutations;

(b) producing the alphavirus particles in the helper cell; and
(c) collecting the alphavirus particles from the helper cells;

30 B) (a) providing a second helper cell for producing a second population of

infectious, replication defective alphavirus particle, comprising in an alphavirus-permissive cell:

(i) an alphavirus replicon RNA, wherein the replicon RNA comprises an alphavirus packaging signal and a nucleic acid encoding a *gag* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, wherein the *gag* gene product or immunogenic fragment thereof is modified to inhibit formation of virus-like particles containing the *gag* gene product or the immunogenic fragment thereof and their release from a cell, and wherein the replicon RNA lacks sequences encoding alphavirus structural proteins;

(ii) a first helper RNA separate from said replicon RNA, said first helper RNA encoding at least one alphavirus structural protein and furthermore not encoding at least one other alphavirus structural protein; and

(iii) one or more additional helper RNA(s) separate from said replicon RNA and separate from said first helper RNA, said additional helper RNA(s) encoding at least one other alphavirus structural protein not encoded by said first helper RNA;

and with at least one of said helper RNAs lacking an alphavirus packaging signal;

wherein the combined expression of the alphavirus replicon RNA and the helper RNAs produces an assembled alphavirus particle which is able to infect a cell, and is unable to complete viral replication, and further wherein the second population contains no detectable replication-competent alphavirus particles as determined by passage on permissive cells in culture, and further wherein at least one of said replicon RNA, said first helper RNA, and said one or more additional helper RNA(s) comprises one or more attenuating mutations;

- (b) producing the alphavirus particles in the helper cell; and
- (c) collecting the alphavirus particles from the helper cells;

C) (a) providing a third helper cell for producing a third population of infectious, replication defective alphavirus particles, comprising in an alphavirus-permissive cell:

5 (i) an alphavirus replicon RNA, wherein the replicon RNA comprises an alphavirus packaging signal and a nucleic acid encoding a *pol* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, wherein the *pol* gene product or immunogenic fragment thereof comprises a modification resulting in deletion or inactivation of integrase, RNase H and reverse transcriptase functions in the *pol* gene product or immunogenic fragment thereof, and wherein the replicon RNA lacks sequences encoding alphavirus structural proteins;

10 (ii) a first helper RNA separate from said replicon RNA, said first helper RNA encoding at least one alphavirus structural protein and furthermore not encoding at least one other alphavirus structural protein; and

15 (iii) one or more additional helper RNA(s) separate from said replicon RNA and separate from said first helper RNA, said additional helper RNA(s) encoding at least one other alphavirus structural protein not encoded by said first helper RNA;

20 and with at least one of said helper RNAs lacking an alphavirus packaging signal;

wherein the combined expression of the alphavirus replicon RNA and the helper RNAs produces an assembled alphavirus particle which is able to infect a cell, and is 25 unable to complete viral replication, and further wherein the third population contains no detectable replication-competent alphavirus particles as determined by passage on permissive cells in culture, and further wherein at least one of said replicon RNA, said first helper RNA, and said one or more additional helper RNA(s) comprises one or more attenuating mutations;

30 (b) producing the alphavirus particles in the helper cell; and

(c) collecting the alphavirus particles from the helper cells; and

5 D) combining the first population of alphavirus particles produced from the first helper cell, the second population of alphavirus particles produced from the second helper cell and the third population of alphavirus particles produced from the third helper cell, thereby producing the population of alphavirus replicon particles.

In each of the methods above, the alphavirus replicon RNA of at least one of the first helper cell, the second helper cell and the third helper cell can comprise
10 sequence encoding at least one alphavirus structural protein and the first helper RNA and the one or more additional helper RNA(s) in the at least one of the first helper cell, the second helper cell and the third helper cell, can encode at least one other alphavirus structural protein not encoded by the replicon RNA.

15 Furthermore, in the methods above which recite attenuating mutations, only at least one of the first population of alphavirus particles, the second population of alphavirus particles and the third population of alphavirus particles can comprise particles wherein at least one of the replicon RNA, the first helper RNA, and the one or more additional helper RNA(s) comprises one or more attenuating mutations.

20

The present invention further provides alphavirus particles produced by any of the methods of this invention.

25 The present invention further provides a method of inducing an immune response to human immunodeficiency virus in a subject, comprising administering to the subject an immunogenic amount of the populations and/or compositions of this invention, in a pharmaceutically acceptable carrier.

30 Also provided herein is a method of treating or preventing infection by human immunodeficiency virus in a subject, comprising administering to the subject an

immunogenic amount of the populations and/or compositions of this invention, in a pharmaceutically acceptable carrier.

Also provided by the present invention is an alphavirus replicon virosome 5 comprising an alphavirus replicon RNA encapsidated by a lipid bilayer comprising alphavirus glycoproteins, E1 and E2, which in one embodiment, can be Venezuelan Equine Encephalitis glycoproteins E1 and E2.

A method of producing an alphavirus replicon virosome is further provided, 10 comprising: a) combining alphavirus replicon RNA, alphavirus glycoproteins E1 and E2, non-cationic lipids and detergent; and b) gradually removing detergent, whereby alphavirus replicon virosomes are produced. Also provided is a virosome produced by this method.

15 Furthermore, the present invention provides a method of eliciting an immune response in a subject, comprising administering to the subject an immunogenic amount of the alphavirus replicon virosome of this invention in a pharmaceutically acceptable carrier.

20 The present invention additionally provides a method of treating or preventing infection by human immunodeficiency virus in a subject, comprising administering to the subject an immunogenic amount of the alphavirus replicon virosome of this invention, wherein the virosome comprises alphavirus replicon RNA encoding one or more HIV immunogens.

25

In further embodiments, the present invention provides a composition a population of alphavirus replicon virosomes comprising two or more isolated nucleic acids selected from the group consisting of 1) an isolated nucleic acid encoding an *env* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, 30 2) an isolated nucleic acid encoding a *gag* gene product or an immunogenic fragment

thereof of a human immunodeficiency virus, wherein the *gag* gene product or immunogenic fragment thereof is modified to inhibit formation of virus-like particles containing the *gag* gene product or the immunogenic fragment thereof and their release from a cell, and 3) an isolated nucleic acid encoding a *pol* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, wherein the *pol* gene product or immunogenic fragment thereof comprises a modification resulting in deletion or inactivation of integrase, RNase H and reverse transcriptase functions in the *pol* gene product or immunogenic fragment thereof, and wherein the nucleic acids are each contained within a separate alphavirus replicon virosome.

10

Additionally provided herein is a composition comprising a population of alphavirus replicon virosomes comprising two or more isolated nucleic acids selected from the group consisting of 1) an isolated nucleic acid encoding an *env* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, 2) an isolated nucleic acid encoding a *gag* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, wherein the *gag* gene product or immunogenic fragment thereof is modified to inhibit formation of virus-like particles containing the *gag* gene product or the immunogenic fragment thereof and their release from a cell, and 3) an isolated nucleic acid encoding a *pol* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, wherein the *pol* gene product or immunogenic fragment thereof comprises a modification resulting in inactivation of reverse transcriptase activity in the *pol* gene product or immunogenic fragment thereof, and wherein the nucleic acids are each contained within a separate alphavirus replicon virosome.

25

A method of producing a population of alphavirus replicon virosomes is provided herein, comprising:

A) (a) producing a first population of alphavirus replicon virosomes by combining alphavirus replicon RNA comprising nucleic acid encoding and *env* gene product or immunogenic fragment thereof, alphavirus glycoproteins E1 and E2, non-

cationic lipids and detergent; and

5 b) gradually removing detergent, whereby alphavirus replicon virosomes are produced;

10

B) (a) producing a second population of alphavirus replicon virosomes by combining alphavirus replicon RNA comprising nucleic acid encoding and *gag* gene product or immunogenic fragment thereof, wherein the *gag* gene product or immunogenic fragment thereof is modified to inhibit formation of virus-like particles containing the *gag* gene product or the immunogenic fragment thereof and their release from a cell, alphavirus glycoproteins E1 and E2, non-cationic lipids and detergent; and

15 b) gradually removing detergent, whereby alphavirus replicon virosomes are produced;

20

C) (a) producing a third population of alphavirus replicon virosomes by combining alphavirus replicon RNA comprising nucleic acid encoding the *pol* gene product or immunogenic fragment thereof, wherein the *pol* gene product or immunogenic fragment thereof comprises a modification resulting in deletion or inactivation of integrase, RNase H and reverse transcriptase functions in the *pol* gene product or immunogenic fragment thereof, alphavirus glycoproteins E1 and E2, non-cationic lipids and detergent; and

25 b) gradually removing detergent, whereby alphavirus replicon virosomes are produced; and

30

D) combining the first population of alphavirus replicon virosomes, the second population of alphavirus replicon virosomes and the third population of alphavirus replicon virosomes to produce the population of alphavirus replicon virosomes.

In addition, a method of producing a population of alphavirus replicon virosomes is provided, comprising:

A) (a) producing a first population of alphavirus replicon virosomes by combining alphavirus replicon RNA comprising nucleic acid encoding and *env* gene product or immunogenic fragment thereof, alphavirus glycoproteins E1 and E2, non-cationic lipids and detergent; and

5 b) gradually removing detergent, whereby alphavirus replicon virosomes are produced;

10

B) (a) producing a second population of alphavirus replicon virosomes by combining alphavirus replicon RNA comprising nucleic acid encoding and *gag* gene product or immunogenic fragment thereof, wherein the *gag* gene product or immunogenic fragment thereof is modified to inhibit formation of virus-like particles

15 containing the *gag* gene product or the immunogenic fragment thereof and their release from a cell, alphavirus glycoproteins E1 and E2, non-cationic lipids and detergent; and

b) gradually removing detergent, whereby alphavirus replicon virosomes are produced;

20

C) (a) producing a third population of alphavirus replicon virosomes by combining alphavirus replicon RNA comprising nucleic acid encoding the *pol* gene product or immunogenic fragment thereof, wherein the *pol* gene product or immunogenic fragment thereof comprises a modification resulting in inactivation of

25 reverse transcriptase activity in the *pol* gene product or immunogenic fragment thereof, alphavirus glycoproteins E1 and E2, non-cationic lipids and detergent; and

b) gradually removing detergent, whereby alphavirus replicon virosomes are produced; and

D) combining the first population of alphavirus replicon virosomes, the second

30 population of alphavirus replicon virosomes and the third population of alphavirus

replicon virosomes to produce the population of alphavirus replicon virosomes of claim 48.

Furthermore, the present invention provides a method of inducing an immune response in a subject, comprising administering to the subject an immunogenic amount of the virosomes of this invention, in a pharmaceutically acceptable carrier.

Also provided is a method of treating or preventing infection by human immunodeficiency virus in a subject, comprising administering to the subject an immunogenic amount of the virosomes of this invention, in a pharmaceutically acceptable carrier.

Additionally provided by this invention is a composition comprising heparin affinity-purified alphavirus replicon particles, wherein the alphavirus replicon particles comprise at least one structural protein which comprises one or more attenuating mutations, as well as a method of preparing heparin affinity-purified alphavirus particles, comprising:

- a) producing alphavirus replicon particles, wherein the alphavirus replicon particles comprise at least one structural protein which comprises one or more attenuating mutations;
- b) loading the alphavirus replicon particles of step (a) in a heparin affinity chromatography column; and
- c) collecting the fraction from the column which contains the heparin affinity-purified alphavirus replicon particles.

25

In further embodiments, the present invention provides a method of producing VRP for use in a vaccine comprising:

- a) producing a plasmid encoding the nucleotide sequence of an alphavirus replicon RNA;
- b) producing a plasmid encoding the nucleotide sequence of one or more

helper RNAs;

- c) transcribing the plasmids of steps (a) and (b) into RNA *in vitro*;
- d) electroporating the RNA of step (c) into a Vero cell line; and

5

- e) purifying VRP from the Vero cell line of step (d) by heparin affinity chromatography. By this method, VRPs can be produced in large-scale.

In additional embodiments, the present invention provides an isolated nucleic acid encoding a *pol* gene product or immunogenic fragment thereof of a human immunodeficiency virus, wherein the *pol* gene product or immunogenic fragment thereof comprises a modification resulting in deletion or inactivation of integrase, RNase H and reverse transcriptase functions in the *pol* gene product or immunogenic fragment thereof. This nucleic acid can be present in a composition and in a vector.

10 Such a vector can be present in a cell. This nucleic acid can also be present in an alphavirus replicon particle.

15

The present invention further provides a method of making an alphavirus replicon particle comprising nucleic acid encoding a *pol* gene product or immunogenic fragment thereof of a human immunodeficiency virus, wherein the *pol* gene product or immunogenic fragment thereof comprises a modification resulting in deletion or inactivation of integrase, RNase H and reverse transcriptase functions in the *pol* gene product or immunogenic fragment thereof, comprising

20

a) providing a helper cell for producing an infectious, defective alphavirus particle, comprising in an alphavirus-permissive cell:

25

- (i) an alphavirus replicon RNA, wherein the replicon RNA comprises an alphavirus packaging signal and a nucleic acid encoding a *pol* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, wherein the *pol* gene product or immunogenic fragment thereof comprises a modification resulting in deletion or

inactivation of integrase, RNase H and reverse transcriptase functions in the *pol* gene product or immunogenic fragment thereof, and wherein the replicon RNA lacks sequences encoding alphavirus structural proteins;

5 (ii) a first helper RNA separate from said replicon RNA, said first helper RNA encoding at least one alphavirus structural protein and furthermore not encoding at least one other alphavirus structural protein; and

10 (iii) one or more additional helper RNA(s) separate from said replicon RNA and separate from said first helper RNA, said additional helper RNA(s) encoding at least one other alphavirus structural protein not encoded by said first helper RNA;

and with at least one of said helper RNAs lacking an alphavirus packaging signal;

wherein the combined expression of the alphavirus replicon RNA and the helper 15 RNAs produces an assembled alphavirus particle which is able to infect a cell, and is unable to complete viral replication, and further wherein the population contains no detectable replication-competent alphavirus particles as determined by passage on permissive cells in culture;

20 (b) producing the alphavirus particles in the helper cell; and
(c) collecting the alphavirus particles from the helper cell.

In the method described above, at least one of the replicon RNA, the first helper RNA, and the one or more additional helper RNA(s) can comprises one or more attenuating mutations. The present invention additionally provides alphavirus 25 replicon particle produced according to the above methods.

Further provided is a method of inducing an immune response in a subject, comprising administering to the subject an immunogenic amount of a composition comprising alphavirus replicon particles encoding a *pol* gene product or an

30 immunogenic fragment thereof of a human immunodeficiency virus, wherein the *pol*

gene product or immunogenic fragment thereof comprises a modification resulting in deletion or inactivation of integrase, RNase H and reverse transcriptase functions in the *pol* gene product or immunogenic fragment thereof in a pharmaceutically acceptable carrier.

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BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. DNA plasmid map of VEE replicon RNA expressing the HIV *gag* gene (p3-40.1.6). The plasmid is 12523 base pairs in length and encodes a single 10 polyprotein expressing the four non-structural genes nsP1-4, the Clade C *gag* gene and antibiotic resistance marker, Kanamycin KN(R). The plasmid contains two promoter regions, the T7 polymerase promoter and the 26S RNA promoter. The unique NotI restriction enzyme site used to linearize prior to *in vitro* transcription is also noted.

Figure 2. DNA plasmid map of the capsid helper construct (p3-13.2.2). The plasmid is 5076 base pairs in length and encodes the VEE capsid gene (C) and antibiotic resistance marker, Kanamycin KN(R). The plasmid contains two promoter regions, the T7 polymerase promoter and the 26S RNA promoter. The unique NotI restriction enzyme site used to linearize prior to *in vitro* transcription is also noted.

20

Figure 3. DNA plasmid map of the glycoprotein helper construct (p3-13.4.6). The plasmid is 6989 base pairs in length and encodes the VEE glycoprotein genes (E3, E2, 6K and E1) and antibiotic resistance marker, Kanamycin KN(R). The plasmid contains two promoter regions, the T7 polymerase promoter and the 26S RNA promoter. The unique NotI restriction enzyme site used to linearize prior to *in vitro* transcription is also noted.

Figure 4. DNA plasmid map of VEE replicon RNA expressing HIV *pol* (p51) gene (p13-60.2.14). The plasmid is 12379 base pairs in length and encodes a single 30 polyprotein expressing the four non-structural genes, nsP1-4, the Clade C *pol* (p51)

gene and antibiotic resistance marker, Kanamycin KN(R). The plasmid contains two promoter regions, the T7 polymerase promoter and the 26S RNA promoter. The unique NotI restriction enzyme site used to linearize prior to *in vitro* transcription is also noted.

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Figure 5. DNA plasmid map of VEE replicon RNA expressing HIV *env* gene (pERK-DU151env). The plasmid is 13584 base pairs in length and encodes a single polyprotein expressing the four non-structural genes, nsP1-4, the Clade C env gene and antibiotic resistance marker, Kanamycin KN(R). The plasmid contains two promoter regions, the T7 polymerase promoter and the 26S RNA promoter. The unique NotI restriction enzyme site used to linearize prior to *in vitro* transcription is also noted.

Figure 6. Western immunoblot, demonstrating the expression of HIV proteins in baby hamster kidney (BHK) cells infected with VRPs. The outer lanes of the panel are standard molecular weight markers. Lane 1 is the expression from VRPs encoding the p51 (*pol*) gene. Lane 2 is the expression from VRPs encoding the GP-160 (*env*) gene. Lane 3 is the expression from VRPs encoding the p55 (*gag*) gene. Arrows indicate proteins migrating with the apparent molecular weight of each respective protein.

20

Figure 7. Western immunoblot of cells infected with the Du151*env* VRP. At 18 hr post infection, the cells were lysed and the lysate run in a denaturing polyacrylamide gel. Proteins were transferred out of the gel onto a filter and the filter was probed with serum from subject Du151 using Western immunoblot methods. Lane 1, uninfected U87.CD4-CXCR4 cells. Lane 2, uninfected U87.CD4-CCR5 cells. Lane 3, infection of a mixed culture of U87.CD4-CXCR4 cells and BHK cells (mixtures were used as a positive control in case the U87 cells were refractory to infection by the VRP, which did not turn out to be the case). Lane 4, infected U87.CD4-CXCR4 cells. Lane 5, infected BHK cells. Lane 6, infection of a mixture of BHK cells and U87.CD4-CCR5 cells. Lane 7, infected U87.CD4-CCR5 cells. The positions of

molecular weight of markers run in the same gel are shown on the right, and the inferred positions of gp160 and gp120 are shown on the left.

Figure 8. Micrographs of U87.CD4-CCR and BHK cells used to examine expression and syncytium formation of DU151 envelope expressed from the VEE replicon. U87.CD4-CCR5 cells alone (panel 1), or a mixture of U87.CD4-CCR5 and BHK cells (Panel 2), BHK cells alone (Panel 3) and U87.CD4-CXCR4 cells (panel 4) were infected with DU151 env VRP at a multiplicity of infection of 3 i.u. per cell. At 18 hours post infection, the cells were examined using light microscopy for the presence of syncytia. The U87.CD4-CCR5 in panel 1 and 2 show clear syncytia, which was absent in the control cell types in the lower panels. In addition, no syncytia were seen in uninfected control cells or VRP-GFP infected cells (data not shown).

Figures 9A-C. Antigen-specific CTL response in mice to the HIV-1 Clade C VRP-gag vaccine. Eight BALB/c mice were immunized twice, first at day 0 and again at day 28 with 10^3 i.u. (Panel A) or 10^5 i.u. (panels B and C) VRP-gag. Eight days (Panels A and B) or 49 days (Panel C) post-boost, spleen cells were isolated and stimulated *in vitro* with vaccinia virus expressing HIV Gag for 1 week. Chromium release assays were performed using vaccinia-Gag infected target cells (diamond symbols) or control vaccinia alone-infected sc11 target cells (square symbols). Clear HIV Gag-specific lysis was detected in animals vaccinated with the VRP-gag vaccine.

Figure 10. Diagrammatic representation of the HIV-1 genome. Black bars indicate relative regions of the genome sequenced to generate phylogenetic sequence comparative data for Clade C *gag*, *pol* and *env* gene isolates.

Figure 11. Phylogenetic comparison of DU422 Clade C Gag isolate with referenced Clade C strains. Consensus clade A, B, D, Mal and SA strains are also shown. DU422 the vaccine strain had 95% amino acid sequence homology to the South African consensus Clade C sequence.

Figure 12. Phylogenetic comparison of DU151 Clade C isolate Env isolate with referenced Clade C strains. DU422 the vaccine strain had 93% amino acid sequence homology to the South African consensus Clade C sequence.

5 Figure 13. Phylogenetic comparison of DU151 Clade C isolate Pol isolate with referenced Clade C strains. DU422 the vaccine strain had 99% amino acid sequence homology to the South African consensus Clade C sequence.

10 Figure 14. DU422 HIV Gag expression as detected by immunofluorescence following electroporation with Gag replicon RNA. BHK cells were electroporated and subjected to immunofluorescence staining with an anti-Gag monoclonal antibody at 24 hours post-electroporation, to demonstrate expression of the Clade C protein.

15 Figure 15. Immunofluorescence detection of DU422 Gag protein expression in BHK cells. BHK cells were infected with VRP-Gag particles and subjected to immunofluorescence staining with an anti-Gag monoclonal antibody at 24 hours post-infection, to demonstrate expression of the Clade C protein.

DETAILED DESCRIPTION OF THE INVENTION

20

As used in the specification and the appended claims, the singular forms "a," "an," and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "a pharmaceutical carrier" can mean a single pharmaceutical carrier or mixtures of two or more such carriers.

25

The present invention is based on the discovery of a vaccine for the treatment and/or prevention of infection by HIV, comprising novel combinations of isolated nucleic acids encoding two or more distinct antigens which elicit an immune response in a subject which is effective in treating and/or preventing infection by HIV. In a 30 particular embodiment, the nucleic acids encoding the antigens of the vaccine are

modified to enhance the immunogenicity of the antigen, improve the safety of the vaccine, or both.

As used herein, the term "isolated nucleic acid" means a nucleic acid separated 5 or substantially free from at least some of the other components of the naturally occurring organism, for example, the cell structural components commonly found associated with nucleic acids in a cellular environment and/or other nucleic acids. The isolation of nucleic acids can be accomplished by well known techniques such as cell lysis or disruption of virus particles, followed by phenol plus chloroform extraction, 10 followed by ethanol precipitation of the nucleic acids (Sambrook *et al.*, latest edition). The nucleic acids of this invention can be isolated according to methods well known in the art for isolating nucleic acids. Alternatively, the nucleic acids of the present invention can be synthesized according to standard protocols well described in the literature for synthesizing nucleic acids.

15

HIV-VRP Vaccines

The antigens of this invention can be gene products which are complete proteins or any fragment of a protein determined to be immunogenic by methods well known in 20 the art. Modifications are made to the antigens of this invention to enhance immunogenicity and/or improve the safety of administration of a vaccine containing the antigen. Examples of such modifications are described in the Examples section herein. Furthermore, it is understood that, where desired, other modifications and changes (e.g., substitutions, deletions, additions) may be made in the amino acid sequence of the 25 antigen of the present invention, which may not specifically impart enhanced immunogenicity or improved safety, yet still result in a protein or fragment which retains all of the functional characteristics by which the protein or fragment is defined. Such changes may occur in natural isolates, may be introduced by synthesis of the protein or fragment, or may be introduced into the amino acid sequence of the protein 30 or fragment using site-specific mutagenesis of nucleic acid encoding the protein or

fragment, the procedures for which, such as mis-match polymerase chain reaction (PCR), are well known in the art.

The nucleic acids of this invention can be present in a vector and the vector of 5 this invention can be present in a cell. The vectors and cells of this invention can be in a composition comprising the cell or vector and a pharmaceutically acceptable carrier.

The vector of this invention can be an expression vector which contains all of 10 the genetic components required for expression of the nucleic acids of this invention in cells into which the vector has been introduced, as are well known in the art. For example, the expression vector of this invention can be a vector comprising the helper RNAs of this invention. Such an expression vector can be a commercial expression vector or it can be constructed in the laboratory according to standard molecular biology protocols. The expression vector can comprise viral nucleic acid including, but 15 not limited to, alphavirus, flavivirus, adenovirus, retrovirus and/or adeno-associated virus nucleic acid. The nucleic acid or vector of this invention can also be in a liposome or a delivery vehicle which can be taken up by a cell via receptor-mediated or other type of endocytosis.

20 In another embodiment, the nucleic acids of this invention can be present in a composition comprising a population of alphavirus replicon particles which comprise two or more distinct isolated nucleic acids of this invention and wherein the nucleic acids are each contained within a separate alphavirus replicon particle (herein referred to as a "VRP"). Thus, the expression vector of the present invention can be an 25 alphavirus replicon particle comprising a nucleic acid encoding an antigen of this invention.

In a particular embodiment, the present invention provides a composition comprising two or more isolated nucleic acids selected from the group consisting of an 30 isolated nucleic acid encoding an *env* gene product or an immunogenic fragment

thereof of a human immunodeficiency virus, an isolated nucleic acid encoding a *gag* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, wherein the *gag* gene product or immunogenic fragment thereof is modified to inhibit formation of particles, e.g., virus-like particles, containing the *gag* gene product or the 5 immunogenic fragment thereof, and their release from a cell, and an isolated nucleic acid encoding a *pol* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, wherein the *pol* gene product or immunogenic fragment thereof is modified to inhibit reverse transcriptase activity.

10 In a preferred embodiment, the invention provides alphavirus replicon particles (VRPs) that can be administered as an HIV vaccine. These HIV-VRPs are propagation defective, single cycle vectors that contain a self-amplifying RNA (replicon RNA, e.g., from VEE, in which the structural protein genes of the virus are replaced by a HIV-1 Clade C *gag* gene or any other HIV antigen to be expressed. Following introduction 15 into packaging (or helper) cells *in vitro*, the replicon RNA is packaged into VRPs by supplying the viral structural proteins in *trans* (helper RNAs).

The present invention further provides a population of alphavirus replicon particles comprising two or more isolated nucleic acids selected from the group 20 consisting of 1) an isolated nucleic acid encoding an *env* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, 2) an isolated nucleic acid encoding a *gag* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, wherein the *gag* gene product or immunogenic fragment thereof is modified to inhibit formation of particles, such as virus-like 25 particles, containing the *gag* gene product or the immunogenic fragment thereof, from a cell, and 3) an isolated nucleic acid encoding a *pol* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, wherein the *pol* gene product or immunogenic fragment thereof is modified to inhibit reverse transcriptase activity, and wherein the nucleic acids are each contained within a separate alphavirus replicon 30 particle.

It is also contemplated that the compositions of this invention comprise alphavirus replicon particles in which either the replicon RNA or at least one structural protein comprises one or more attenuating mutations. Thus, the present invention additionally provides a population of alphavirus replicon particles comprising two or

5 more distinct types of such particles selected from the group consisting of 1) particles expressing a nucleic acid encoding an *env* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, 2) particles expressing a nucleic acid encoding a *gag* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, wherein the *gag* gene product or immunogenic fragment

10 thereof is modified to inhibit release of particles, such as virus-like particles, containing the *gag* gene product or the immunogenic fragment thereof, from a cell, and 3) particles expressing a nucleic acid encoding a *pol* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, wherein the *pol* gene product or immunogenic fragment thereof is modified to inhibit reverse transcriptase activity; and

15 wherein the nucleic acids are each contained within a separate alphavirus replicon particle and further wherein the alphavirus replicon particles comprise a replicon RNA or at least one structural protein which comprises one or more attenuating mutations.

In a preferred embodiment, the population of alphavirus replicon particles

20 comprises particles expressing the nucleic acids encoding *pol*, *env*, and *gag* gene products. In this embodiment, vigorous antigen-specific cellular (e.g., CTL, NK cell and T-helper) and/or humoral (e.g., antibody) responses can be obtained when such particle populations are administered to a subject.

25 In the compositions described above, the *gag* gene product or immunogenic fragment thereof can be modified by mutation of the second codon, whereby a glycine is changed to an alanine. Alternatively, the *gag* gene product or immunogenic fragment thereof can be modified by any other means known in the art for inhibiting the release of particles containing the *gag* gene product or immunogenic fragment

30 thereof from a cell.

Furthermore, in the compositions of this invention, the *pol* gene product or immunogenic fragment thereof can be modified by mutation of the nucleotide sequence encoding the active site motif, whereby YMDD is changed to YMAA or HMAA (the latter providing a convenient site for cloning, see SEQ ID NO:16). The *pol* gene product or immunogenic fragment thereof can also be modified by any means known in the art for inhibiting reverse transcriptase activity.

The *pol* gene product or immunogenic fragment thereof of this invention may be further modified such that the coding sequences for integrase and RNase H are removed, inactivated and/or modified, e.g., by producing only the p51 region of the *pol* gene product. This modification has been shown in some studies to reduce the possibility of formation of replication competent alphavirus particles during production of alphavirus replicon particles comprising the *pol* gene product or immunogenic fragment thereof. This modification can be of the nucleic acid encoding the *pol* gene product or immunogenic fragment thereof according to methods known in the art. Thus, the particles and compositions of this invention can comprise nucleic acid encoding a *pol* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, wherein the *pol* gene product or immunogenic fragment thereof comprises a modification resulting in deletion or inactivation of integrase, RNase H and reverse transcriptase functions in the *pol* gene product or immunogenic fragment thereof.

In the compositions of this invention, the *gag*, *env* or *pol* gene products or immunogenic fragments thereof can be from any HIV isolate or consensus sequence derived from HIV primary isolates now known or later identified, the isolation and characterization of which are well known in the art. Also, in the compositions of this invention, the *gag*, *env* or *pol* gene products or immunogenic fragments thereof can be produced from the same HIV isolate or HIV consensus sequence or from any combination of HIV isolates or HIV consensus sequences. In the Examples provided herein, the nucleic acid sequences encoding the *env*, *gag* and *pol* gene products of this

invention were selected based on a consensus sequence generated from primary isolates obtained from recent seroconvertors in Kwazulu/Natal in South Africa. Sequence analysis of these isolates identified them as subtype (or clade) C, and in preferred embodiments of the invention, the *env*, *gag* and *pol* genes are from Clade C isolates of 5 HIV.

In preferred embodiments, each of the three HIV genes are derived from one or more of the South African isolates obtained from recent seroconverters in Kwazulu/Natal as described herein (see Figures 11-13 for isolate names). In a further 10 embodiment, the *gag* gene or gene fragment is from a *gag* sequence having 95% or greater amino acid identity with the South African consensus sequence for the *gag* gene. In a specific embodiment, the *gag* gene or fragment thereof is derived from HIV Subtype Clade C isolate DU422 and the *env* and *pol* genes or fragments thereof are derived from HIV isolate DU151.

15 The term "alphavirus" has its conventional meaning in the art and includes the various species of the alphavirus genus, such as Eastern Equine Encephalitis virus (EEE), Venezuelan Equine Encephalitis virus (VEE), Western Equine Encephalitis virus (WEE), Everglades virus, Mucambo virus, Pixuna virus, Sindbis virus, Semliki 20 Forest virus, South African Arbovirus No. 86, Middleburg virus, Chikungunya virus, O=nyong-nyong virus, Ross River virus, Barmah Forest virus, Getah virus, Sagiyama virus, Bebaru virus, Mayaro virus, Una virus, Aura virus, Whataroa virus, Babanki virus, Kyzylagach virus, Highlands J virus, Fort Morgan virus, Ndumu virus, Buggy Creek virus, as well as any specific strains of these alphaviruses (e.g., TR339; 25 Girdwood) and any other virus classified by the International Committee on Taxonomy of Viruses (ICTV) as an alphavirus.

30 An "alphavirus replicon particle" as used herein is an infectious, replication defective, alphavirus particle which comprises alphavirus structural proteins and further comprises a replicon RNA. The replicon RNA comprises nucleic acid encoding the

alphavirus packaging segment, nucleic acid encoding alphavirus non-structural proteins and a heterologous nucleic acid sequence encoding an antigen of this invention. The non-structural proteins encoded by the replicon RNA may be such proteins as are required for replication and transcription. In a specific embodiment of this invention, 5 the structure of the replicon RNA, starting at the 5' end, comprises the 5' untranslated region of the alphavirus RNA, the non-structural proteins (e.g., nsPs1-4) of the alphavirus, the 26S promoter (also known as the "subgenomic promoter"), the heterologous nucleic acid encoding an HIV antigen, and the 3' untranslated region of the alphavirus RNA. An example of a nucleic acid encoding alphavirus nonstructural 10 proteins that can be incorporated into the embodiments of this invention is SEQ ID NO:2, which encodes the amino acid sequence of SEQ ID NO:3.

Although the alphavirus replicon RNA can comprise nucleic acid encoding one or two alphavirus structural proteins, the replicon RNA does not contain nucleic acid 15 encoding all of the alphavirus structural proteins. The replicon RNA can lack nucleic acid encoding any alphavirus structural protein(s). Thus, the resulting alphavirus replicon particles of this invention are replication defective inasmuch as the replicon RNA does not encode all of the structural proteins required for encapsidation of the replicon RNA and assembly of an infectious virion.

20

As used herein, "alphavirus structural protein" or "structural protein" means the alphavirus proteins required for encapsidation of alphavirus replicon RNA and packaging of the encapsidated RNA into a virus particle. The alphavirus structural proteins include PE2, E2, E3, 6K and E1.

25

The alphavirus replicon particles of this invention can comprise replicon RNA from any of the alphaviruses of this invention. Furthermore, the alphavirus replicon particles of this invention can comprise alphavirus structural proteins from any of the alphaviruses of this invention. Thus, the replicon particles can be made up of replicon 30 RNA and structural proteins from the same alphavirus or from different alphaviruses,

the latter of which would be chimeric alphavirus replicon particles (e.g., a particle comprising Sindbis virus replicon RNA and VEE structural proteins).

The alphavirus replicon particles of this invention can be made by employing a

5 helper cell for expressing an infectious, replication defective, alphavirus particle in an alphavirus-permissive cell. The helper cell includes (a) a first helper RNA encoding (i) at least one alphavirus structural protein, and (ii) not encoding at least one alphavirus structural protein; and (b) a second helper RNA separate from the first helper RNA, the second helper RNA (i) not encoding the at least one alphavirus structural protein

10 encoded by the first helper RNA, and (ii) encoding at least one alphavirus structural protein not encoded by the first helper RNA, such that all of the alphavirus structural proteins assemble together into alphavirus particles in the cell.

The alphavirus structural protein genes can be present on the helper RNAs of this invention in any combination. For example, the helper RNA of this invention can

15 encode the alphavirus capsid and E1, capsid and E2, E1 and E2, capsid only, E1 only, E2 only, etc. It is also contemplated that the alphavirus structural proteins are provided in *trans* from genes located on three separate RNA molecules within the helper cell.

In a preferred embodiment, the helper cell also includes a replicon RNA, which

20 encodes the alphavirus packaging segment and an inserted heterologous RNA. In the embodiment wherein the helper cell also includes a replicon RNA, the alphavirus packaging segment may be, and preferably is, deleted from both the first helper RNA and the second helper RNA. For example, in an embodiment wherein the helper cell includes a replicon RNA encoding the alphavirus packaging segment and an inserted

25 heterologous RNA, the first helper RNA encodes the alphavirus E1 glycoprotein and the alphavirus E2 glycoprotein, and the second helper RNA encodes the alphavirus capsid protein. In a preferred embodiment, the first helper RNA encodes the E3-E2-6k-E1 cassette from an alphavirus. In an alternative embodiment, the cassette encoded on the first helper RNA is referred to as the E3-E2-E1 cassette. A specific embodiment of

30 this aspect of the invention is diagrammed in Figure 3, and an exemplary nucleotide

sequence is SEQ ID NO:11. The replicon RNA, first helper RNA, and second helper RNA are all on separate molecules and are cotransfected, e.g., by electroporation, into the helper cell, which can be any alphavirus permissive cell, as is well known in the art.

5 In an alternative embodiment, the helper cell includes a replicon RNA encoding the alphavirus packaging segment and an inserted heterologous RNA and also includes the alphavirus capsid protein otherwise encoded by the second helper RNA. The first helper RNA encodes the alphavirus E1 glycoprotein and the alphavirus E2 glycoprotein. Thus, the replicon RNA and the first helper RNA are on separate 10 molecules, and the replicon RNA and the second helper RNA are on a single molecule.

15 The RNA encoding the structural proteins, i.e., the first helper RNA and the second helper RNA, can include one or more attenuating mutations. In a preferred embodiment, either one or both of the first helper RNA and the second helper RNA include at least one attenuating mutation. The attenuating mutations provide the advantage that in the event of RNA recombination within the cell, the coming together of the structural and non-structural genes will produce a virus of decreased virulence.

20 The alphavirus replicon particles of this invention can be made by a) transfecting a helper cell as given above with a replication defective replicon RNA, b) producing the alphavirus particles in the transfected cell, and c) collecting the alphavirus particles from the cell. The replicon RNA encodes the alphavirus packaging segment and a heterologous RNA. The transfected helper cell further includes the first 25 helper RNA and second helper RNA as described above.

As described hereinabove, the structural proteins used to assemble the alphavirus replicon particles of this invention are distributed among one or more helper RNAs (i.e., a first helper RNA and a second helper RNA). As noted herein, one or 30 more structural protein genes may be located on the replicon RNA, provided that at

structural protein gene is deleted from the replicon RNA such that the replicon and resulting alphavirus particle are replication defective. As used herein, the "deleted" or "deletion" mean either total deletion of the specified nucleic acid or deletion of a sufficient portion of the specified nucleic acid to render the nucleic acid and/or its resultant gene product inoperative or nonfunctional, in accordance with conventional usage. (See, e.g., U.S. Pat. No. 4,650,764 to Temin *et al.*) The term "replication defective" as used herein means that the replicon RNA cannot replicate in a cell (i.e., produce infectious viral particles) in the absence of the helper RNA. The replicon RNA is replication defective inasmuch as the replicon RNA does not contain all of the alphavirus structural protein genes required for replication, at least one required structural protein genes being deleted therefrom.

In one embodiment, the packaging segment or "encapsidation sequence" is deleted from at least the first helper RNA. In a preferred embodiment, the packaging segment is deleted from both the first helper RNA and the second helper RNA. In a more preferred embodiment, the second helper RNA is constructed from a VEE cDNA clone, containing all non-structural proteins (i.e., nsPs1-4), the packaging signal, and the structural protein cassette (E3-E2-E1). An example of a plasmid encoding such a second helper RNA is provided in Figure 2, and an exemplary nucleotide sequence for such a second helper RNA is SEQ ID NO:8.

In the preferred embodiment wherein the packaging segment is deleted from both the first helper RNA and the second helper RNA, preferably the helper cell contains a replicon RNA in addition to the first helper RNA and the second helper RNA. The replicon RNA encodes the packaging segment and an inserted heterologous gene coding an HIV antigen or a fragment thereof. Typically, the inserted heterologous RNA encodes a gene product which is expressed by the target cell, and contains the promoter and regulatory segments necessary for the expression of that gene product in that cell.

In another preferred embodiment, the replicon RNA, the first helper RNA and the second helper RNA are provided on separate molecules such that a first molecule, i.e., the replicon RNA, encodes the packaging segment and the inserted heterologous RNA, a second molecule, i.e., the first helper RNA, encodes at least one but not all of

- 5 the required alphavirus structural proteins, and a third molecule, i.e., the second helper RNA, encodes at least one but not all of the required alphavirus structural proteins. For example, in one preferred embodiment of the present invention, the helper cell includes a set of RNAs which include (a) a replicon RNA encoding an alphavirus packaging sequence and an inserted heterologous RNA, (b) a first helper RNA encoding the alphavirus E1 glycoprotein and the alphavirus E2 glycoprotein, and (c) a second helper RNA encoding the alphavirus capsid protein, so that the alphavirus E1 glycoprotein, the alphavirus E2 glycoprotein and the capsid protein assemble together into alphavirus particles containing the replicon RNA in the helper cell.

15 In an alternate embodiment, the replicon RNA and the first helper RNA are on separate molecules, and the replicon RNA and the second helper RNA are on a single molecule together, thereby providing a first molecule, i.e., the first helper RNA, encoding at least one but not all of the required alphavirus structural proteins, and a second molecule, i.e., the replicon RNA and second helper RNA, encoding the

- 20 packaging segment, the inserted heterologous gene product and the structural protein(s) not encoded by the first helper. Thus, one or more structural protein(s) is encoded by the second helper RNA, but the second helper RNA is located on the second molecule together with the replicon RNA. For example, in one preferred embodiment of the present invention, the helper cell includes a set of RNAs including (a) a replicon RNA encoding an alphavirus packaging sequence, an inserted heterologous RNA, and an alphavirus capsid protein, and (b) a first helper RNA encoding the alphavirus E1 glycoprotein and the alphavirus E2 glycoprotein so that the alphavirus E1 glycoprotein, the alphavirus E2 glycoprotein and the capsid protein assemble together into alphavirus particles in the helper cell.

The present invention also contemplates alphavirus replicon particles which comprise replicon RNA encoding more than one heterologous gene product. For expression of more than one heterologous nucleic acid from a single replicon RNA, a promoter can be inserted upstream of each heterologous nucleic acid on the replicon 5 RNA, such that the promoter regulates expression of the heterologous nucleic acid, resulting in the production of more than one antigen from a single replicon RNA. Another embodiment contemplates the insertion of an IRES sequence, such as the one from the picornavirus, EMC virus, between the heterologous genes downstream from a 26S promoter of the replicon, thus leading to translation of multiple antigens from a 10 single replicon.

In one preferred embodiment of the present invention, the RNA encoding the alphavirus structural proteins, i.e., the capsid, E1 glycoprotein and/or E2 glycoprotein, 15 contains at least one attenuating mutation. It is further contemplated that the RNA encoding the non-structural proteins can contain at least one attenuating mutation. The phrases "attenuating mutation" and "attenuating amino acid," as used herein, mean a nucleotide mutation or an amino acid coded for in view of such a mutation which result in a decreased probability of causing disease in its host (i.e., a loss of virulence), in 20 accordance with standard terminology in the art, See, e.g., Davis *et al.* (1980). The mutation can be, for example, a substitution mutation or an in-frame deletion mutation. The phrase "attenuating mutation" excludes mutations which would be lethal to the virus. Thus, according to this embodiment, the E1 RNA and/or the E2 RNA and/or the capsid RNA can include at least one attenuating mutation. In a more preferred 25 embodiment, the E1 RNA and/or the E2 RNA and/or the capsid RNA includes at least two, or multiple, attenuating mutations. The multiple attenuating mutations may be positioned in either the first helper RNA or in the second helper RNA, or they may be distributed randomly with one or more attenuating mutations being positioned in the first helper RNA and one or more attenuating mutations positioned in the second helper 30 RNA. Appropriate attenuating mutations will be dependent upon the alphavirus used,

as is well known in the art.

For example, when the alphavirus is VEE, suitable attenuating mutations can be in codons at E2 amino acid position 76 which specify an attenuating amino acid,

- 5 codons at E2 amino acid position 120 which specify an attenuating amino acid, preferably lysine as E2 amino acid 120; codons at E2 amino acid position 209 which specify an attenuating amino acid, preferably lysine, arginine, or histidine as E2 amino acid 209; codons at E1 amino acid 272 which specify an attenuating mutation, preferably threonine or serine as E1
- 10 amino acid 272; codons at E1 amino acid 81 which specify an attenuating mutation, preferably isoleucine or leucine as E1 amino acid 81; and codons at E1 amino acid 253 which specify an attenuating mutation, preferably serine or threonine as E1 amino acid 253; and the combination mutation of the deletion of E3 codons 56-59 together with codons at E1 amino acid 253 which specify an attenuating mutation, as provided
- 15 herein. Other suitable attenuating mutations within the VEE genome will be known to those skilled in the art.

In an alternate embodiment, wherein the alphavirus is the South African Arbovirus No. 86 (S.A.A.R.86), suitable attenuating mutations can be, for example, in

- 20 codons at nsP1 amino acid position 538 which specify an attenuating amino acid, preferably isoleucine as nsP1 amino acid 538; codons at E2 amino acid position 304 which specify an attenuating amino acid, preferably threonine as E2 amino acid 304; codons at E2 amino acid position 314 which specify an attenuating amino acid, preferably lysine as E2 amino acid 314; codons at E2 amino acid position 376 which
- 25 specify an attenuating amino acid, preferably alanine as E2 amino acid 376; codons at E2 amino acid position 372 which specify an attenuating amino acid, preferably leucine as E2 amino acid 372; codons at nsP2 amino acid position 96 which specify an attenuating amino acid, preferably glycine as nsP2 amino acid 96; codons at nsP2 amino acid position 372 which specify an attenuating amino acid, preferably valine as
- 30 nsP2 amino acid 372; in combination, codons at E2 amino acid residues 304, 314, 372

and 376; codons at E2 amino acid position 378 which specify an attenuating amino acid, preferably leucine as E2 amino acid 378; codons at nsP2 amino acid residue 372 which specify an attenuating mutation, preferably valine as nsP2 amino acid 372; in combination, codons at nsP2 amino acid residues 96 and 372 attenuating substitution 5 mutations at nsP2 amino acid residues 96 and 372; codons at nsP2 amino acid residue 529 which specify an attenuating mutation, preferably leucine, at nsP2 amino acid residue 529; codons at nsP2 amino acid residue 571 which specify an attenuating mutation, preferably asparagine, at nsP2 amino acid residue 571; codons at nsP2 amino acid residue 682 which specify an attenuating mutation, preferably arginine, at nsP2 10 amino acid residue 682; codons at nsP2 amino acid residue 804 which specify an attenuating mutation, preferably arginine, at nsP2 amino acid residue 804; codons at nsP3 amino acid residue 22 which specify an attenuating mutation, preferably arginine, at nsP3 amino acid residue 22; and in combination, codons at nsP2 amino acid residues 529, 571, 682 and 804, and at nsP3 amino acid residue 22, specifying attenuating 15 amino acids at nsP2 amino acid residues 529, 571, 682 and 804 and at nsP3 amino acid residue 22. Other suitable attenuating mutations within the S.A.A.R.86 genome will be known to those skilled in the art.

The alphavirus capsid gene used to make alphavirus replicon particles can also 20 be subjected to site-directed mutagenesis. The altered capsid protein provides additional assurance that recombination to produce the virulent virus will not occur. The altered capsid protein gene which functions in particle assembly but not in autoproteolysis provides helper function for production of replicon particles, but does not allow for production of a viable recombinant. The capsid residues required for 25 proteolytic function are known (Strauss *et al.*, 1990).

Suitable attenuating mutations useful in embodiments wherein any of the alphaviruses of this invention are employed are known to or can be identified by those skilled in the art using routine protocols. Attenuating mutations may be introduced into 30 the RNA by performing site-directed mutagenesis on the cDNA which encodes the

RNA, in accordance with known procedures. See Kunkel (1985), the disclosure of which is incorporated herein by reference in its entirety. Alternatively, mutations may be introduced into the RNA by replacement of homologous restriction fragments in the cDNA which encodes for the RNA, in accordance with known procedures. The 5 identification of a particular mutation in an alphavirus as attenuating is done using routine experimentation according to methods well known in the art.

Preferably, the helper RNA of this invention includes a promoter. It is also preferred that the replicon RNA includes a promoter. Suitable promoters for inclusion 10 in the helper RNA and replicon RNA are well known in the art. One preferred promoter is the alphavirus 26S promoter, although many suitable promoters are available, as is well known in the art.

In the system wherein a first helper RNA, a second helper RNA, and a replicon 15 RNA are all on separate molecules, if the same promoter is used for all three RNAs, then a homologous sequence between the three molecules is provided. Thus, it is advantageous to employ different promoters on the first and second helper RNAs to provide further impediment to RNA recombination that might produce virulent virus. It is preferred that the selected promoter is operative with the non-structural proteins 20 encoded by the replicon RNA molecule.

The infectious, replication defective, alphavirus particles of this invention are prepared according to the methods disclosed herein in combination with techniques known to those skilled in the art. The methods include, for example, transfecting an 25 alphavirus-permissive cell with a replication defective replicon RNA including the alphavirus packaging segment and an inserted heterologous RNA, a first helper RNA encoding at least one alphavirus structural protein, and a second helper RNA encoding at least one alphavirus structural protein which is different from that encoded by the first helper RNA; producing the alphavirus particles in the transfected cell; and 30 collecting the alphavirus particles from the cell.

Methods for transfecting the alphavirus-permissive cell with the replicon RNA and helper RNAs can be achieved, for example, by (i) treating the cells with

DEAE-dextran, (ii) by lipofection, by treating the cells with, for example,

LIPOFECTIN, and (iii) by electroporation, with electroporation being a preferred

5 means of achieving RNA uptake into the alphavirus-permissive cells. Examples of these techniques are well known in the art, see e.g., U.S. Pat. No. 5,185,440 to Davis *et al.*, and PCT Publication No. WO 92/10578 to Biooption AB, the disclosures of which are incorporated herein by reference in their entirety.

10 The steps of producing the infectious viral particles in the cells may also be carried out using conventional techniques. See e.g., U.S. Patent No. 5,185,440 to Davis *et al.*, PCT Publication No. WO 92/10578 to Biooption AB, and U.S. Patent No. 4,650,764 to Temin *et al.* (although Temin *et al.*, relates to retroviruses rather than alphaviruses). The infectious viral particles may be produced by standard cell culture

15 growth techniques.

The steps of collecting the infectious alphavirus particles may also be carried out using conventional techniques. For example, the infectious particles may be collected by cell lysis, or collection of the supernatant of the cell culture, as is known in

20 the art. See e.g., U.S. Patent No. 5,185,440 to Davis *et al.*, PCT Publication No. WO 92/10578 to Biooption AB, and U.S. Patent No. 4,650,764 to Temin *et al.* (although Temin *et al.* relates to retroviruses rather than alphaviruses). Other suitable techniques will be known to those skilled in the art. Optionally, the collected infectious alphavirus particles may be purified, if desired. Purification techniques for viruses are well known

25 to those skilled in the art, and these are suitable for the purification of small batches of infectious alphavirus particles.

Thus, the present invention provides a method of making the populations of alphavirus replicon particles of this invention comprising:

30 A) (a) providing a first helper cell for producing a first population of infectious,

defective alphavirus particles, comprising in an alphavirus-permissive cell:

- (i) an alphavirus replicon RNA, wherein the replicon RNA comprises an alphavirus packaging signal and a nucleic acid encoding an *env* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, and wherein the replicon RNA lacks sequences encoding alphavirus structural proteins;
- (ii) a first helper RNA separate from said replicon RNA, said first helper RNA encoding at least one alphavirus structural protein and furthermore not encoding at least one other alphavirus structural protein; and
- (iii) one or more additional helper RNA(s) separate from said replicon RNA and separate from said first helper RNA, said additional helper RNA(s) encoding at least one other alphavirus structural protein not encoded by said first helper RNA;

and with at least one of said helper RNAs lacking an alphavirus packaging signal;

wherein the combined expression of the alphavirus replicon RNA and the helper RNAs produces an assembled alphavirus particle which is able to infect a cell, and is unable to complete viral replication, and further wherein the first population contains no detectable replication-competent alphavirus particles as determined by passage on permissive cells in culture;

- (b) producing the alphavirus particles in the helper cell; and
- (c) collecting the alphavirus particles from the helper cells;

B) (a) providing a second helper cell for producing a second population of infectious, defective alphavirus particles, comprising in an alphavirus-permissive cell:

- (i) an alphavirus replicon RNA, wherein the replicon RNA comprises an alphavirus packaging signal and a nucleic acid encoding a *gag* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, wherein the *gag* gene product or immunogenic fragment thereof is modified to inhibit formation of virus-like particles

containing the *gag* gene product or the immunogenic fragment thereof and their release from a cell, and wherein the replicon RNA lacks sequences encoding alphavirus structural proteins;

(ii) a first helper RNA separate from said replicon RNA, said first helper RNA encoding at least one alphavirus structural protein and furthermore not encoding at least one other alphavirus structural protein; and

(iii) one or more additional helper RNA(s) separate from said replicon RNA and separate from said first helper RNA, said additional helper RNA(s) encoding at least one other alphavirus structural protein not encoded by said first helper RNA;

and with at least one of said helper RNAs lacking an alphavirus packaging signal;

15 wherein the combined expression of the alphavirus replicon RNA and the helper RNAs produces an assembled alphavirus particle which is able to infect a cell, and is unable to complete viral replication, and further wherein the second population contains no detectable replication-competent alphavirus particles as determined by passage on permissive cells in culture;

20 (b) producing the alphavirus particles in the helper cell; and

(c) collecting the alphavirus particles from the helper cells;

C) providing a third helper cell for producing a third population of infectious, defective alphavirus particles, comprising in an alphavirus-permissive cell:

25 (i) an alphavirus replicon RNA, wherein the replicon RNA comprises an alphavirus packaging signal and a nucleic acid encoding a *pol* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, wherein the *pol* gene product or immunogenic fragment thereof is modified to inhibit reverse transcriptase activity or is modified to inactivate or delete integrase, RNase H and reverse transcriptase functions in the *pol* gene product or immunogenic fragment

thereof, and wherein the replicon RNA lacks sequences encoding alphavirus structural proteins;

(ii) a first helper RNA separate from said replicon RNA, said first helper RNA encoding at least one alphavirus structural protein and furthermore not encoding at least one other alphavirus structural protein; and

(iii) one or more additional helper RNA(s) separate from said replicon RNA and separate from said first helper RNA, said additional helper RNA(s) encoding at least one other alphavirus structural protein not encoded by said first helper RNA;

10 and with at least one of said helper RNAs lacking an alphavirus packaging signal;

wherein the combined expression of the alphavirus replicon RNA and the helper RNAs produces an assembled alphavirus particle which is able to infect a cell, and 15 unable to complete viral replication, and further wherein the third population contains no detectable replication-competent alphavirus particles as determined by passage on permissive cells in culture;

(b) producing the alphavirus particles in the helper cell; and

(c) collecting the alphavirus particles from the helper cells; and

20 D) combining the first population of alphavirus particles produced from the first helper cell, the second population of alphavirus particles produced from the second helper cell and the third population of alphavirus particles produced from the third helper cell, thereby producing the populations of alphavirus replicon particles.

25 In a preferred embodiment, as noted above, the method provided also includes a mutation in the *pol* gene product or immunogenic fragment thereof resulting in inactivation or deletion of integrase and RNase H functions of the *pol* gene product or immunogenic fragment thereof. In a specific embodiment of this method, the region of the *pol* gene encoding the RNase H and integrase function of the *pol* gene product or 30 immunogenic fragment thereof has been deleted.

A method of making the populations of alphavirus replicon particles of this invention, wherein the particles comprise at least one attenuating mutation, is also provided, comprising:

5 A) (a) providing a first helper cell for producing a first population of infectious, defective alphavirus particles, comprising in an alphavirus-permissive cell:

10 (i) an alphavirus replicon RNA, wherein the replicon RNA comprises an alphavirus packaging signal and a nucleic acid encoding an *env* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, and wherein the replicon RNA lacks sequences encoding alphavirus structural proteins;

15 (ii) a first helper RNA separate from said replicon RNA, said first helper RNA encoding at least one alphavirus structural protein and furthermore not encoding at least one other alphavirus structural protein; and

20 (iii) one or more additional helper RNA(s) separate from said replicon RNA and separate from said first helper RNA, said additional helper RNA(s) encoding at least one other alphavirus structural protein not encoded by said first helper RNA;

and with at least one of said helper RNAs lacking an alphavirus packaging

25 signal;

wherein the combined expression of the alphavirus replicon RNA and the helper RNAs produces an assembled alphavirus particle which is able to infect a cell, and is unable to complete viral replication, and further wherein the first population contains no detectable replication-competent alphavirus particles as determined by passage on permissive cells in culture, and further wherein at least one of said replicon RNA, said first helper RNA, and said one or more additional helper RNA(s) comprises one or more attenuating mutations;

30 (b) producing the alphavirus particles in the helper cell; and

(c) collecting the alphavirus particles from the helper cells;

35 B) providing a second helper cell for producing a second population of infectious,

defective alphavirus particles, comprising in an alphavirus-permissive cell:

- (i) an alphavirus replicon RNA, wherein the replicon RNA comprises an alphavirus packaging signal and a nucleic acid encoding a *gag* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, wherein the *gag* gene product or immunogenic fragment thereof is modified to inhibit release of particles, such as virus-like particles, containing the *gag* gene product or the immunogenic fragment thereof from a cell, and wherein the replicon RNA lacks sequences encoding alphavirus structural proteins;
- 5 (ii) a first helper RNA separate from said replicon RNA, said first helper RNA encoding at least one alphavirus structural protein and furthermore not encoding at least one other alphavirus structural protein; and
- 10 (iii) one or more additional helper RNA(s) separate from said replicon RNA and separate from said first helper RNA, said additional helper RNA(s) encoding at least one other alphavirus structural protein not encoded by said first helper RNA;
- 15 and with at least one of said helper RNAs lacking an alphavirus packaging signal;
- 20 wherein the combined expression of the alphavirus replicon RNA and the helper RNAs produces an assembled alphavirus particle which is able to infect a cell, and is unable to complete viral replication, and further wherein the second population contains no detectable replication-competent alphavirus particles as determined by passage on permissive cells in culture, and further wherein at least one of said replicon RNA, said first helper RNA, and said one or more additional helper RNA(s) comprises one or 25 more attenuating mutations;
- (b) producing the alphavirus particles in the helper cell; and
- (c) collecting the alphavirus particles from the helper cells;

C) providing a third helper cell for producing a third population of infectious, 30 defective alphavirus particles, comprising in an alphavirus-permissive cell:

(i) an alphavirus replicon RNA, wherein the replicon RNA comprises an alphavirus packaging signal and a nucleic acid encoding a *pol* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, wherein the *pol* gene product or immunogenic fragment thereof is modified to inhibit reverse transcriptase activity or is modified to inactivate or delete integrase, RNase H and reverse transcriptase functions in the *pol* gene product or immunogenic fragment thereof, and wherein the replicon RNA lacks sequences encoding alphavirus structural proteins;

(ii) a first helper RNA separate from said replicon RNA, said first helper RNA encoding at least one alphavirus structural protein and furthermore not encoding at least one other alphavirus structural protein; and

(iii) one or more additional helper RNA(s) separate from said replicon RNA and separate from said first helper RNA, said additional helper RNA(s) encoding at least one other alphavirus structural protein not encoded by said first helper RNA;

and with at least one of said helper RNAs lacking an alphavirus packaging signal;

wherein the combined expression of the alphavirus replicon RNA and the helper RNAs produces an assembled alphavirus particle which is able to infect a cell, and is unable to complete viral replication, and further wherein the third population contains no detectable replication-competent alphavirus particles as determined by passage on permissive cells in culture, and further wherein at least one of said replicon RNA, said first helper RNA, and said one or more additional helper RNA(s) comprises one or more attenuating mutations;

(b) producing the alphavirus particles in the helper cell; and

(c) collecting the alphavirus particles from the helper cells; and

D) combining the first population of alphavirus particles produced from the first helper cell, the second population of alphavirus particles produced from the second

helper cell and the third population of alphavirus particles produced from the third helper cell, thereby producing the populations of alphavirus replicon particles of the present invention comprising at least one attenuating mutation.

5 In a preferred embodiment, as noted above, the method provided above can include a further mutation in the *pol* gene product or immunogenic fragment thereof resulting in inactivation or deletion of integrase and RNase H functions of the *pol* gene product or immunogenic fragment thereof. In a specific embodiment of this method, the region of the *pol* gene encoding the RNase H and integrase function of the *pol* gene 10 product or immunogenic fragment thereof has been deleted.

It is also contemplated regarding the method described above, that not all of the first, second and third populations of alphavirus particles do not all have to comprise an attenuating mutation. For example, the first population may comprise attenuating 15 mutations, but the second and third populations may not, etc.

The present invention further provides the compositions of the present invention which are produced by the methods of this invention.

20 The compositions and methods of this invention which incorporate attenuating mutations into the alphavirus replicon particles forming the composition and/or produced by the methods include purified compositions and methods of purification based on the presence of the attenuating mutations. In particular, certain attenuating mutations in the alphavirus structural proteins introduce heparin binding sites into these 25 proteins which are present on the surface of the alphavirus replicon particles. As an example, the V3014 E2 glycoprotein (SEQ ID NO:12 and SEQ ID NO:13) has a mutation in which a lysine is substituted for the glutamic acid at amino acid position 209. This mutation, which creates a more positively charged glycoprotein, increases the affinity of this protein for heparin. Thus, it is possible to purify such particles using 30 heparin affinity chromatography. Such chromatography can be performed using any of

several commercially available resins to which heparin has been bound. The source of heparin is variable; the commercially available resins currently use porcine heparin. The choice of resin will be based on its relative ease of use in a scaled-up, GMP-compliant process, e.g., price, column packing limitations, and potential for easy 5 sanitization. The use of heparin affinity chromatography results in a substantial purification of the VRPs with very little loss of material, and it is a scalable purification step. In a preferred embodiment, a heparin affinity chromatography step results in between an 8- to 27-fold reduction in total protein per ml, or from a 300- to 1000-fold reduction in total protein per VRP. Thus, the present invention provides heparin 10 affinity-purified alphavirus replicon particles containing attenuating mutations which are useful as clinical trial material and commercial product. The present invention also provides methods for preparing purified alphavirus replicon particles containing attenuating mutations comprising the use of heparin affinity chromatography, as described in the Examples provided herein. These particles can also be present in a 15 composition of this invention.

The alphavirus replicon particles of this invention can also be made in a cell free system. Such replicon particles are herein referred to as virosomes. In a specific embodiment of the method, such particles are constructed from a mixture containing 20 replicon RNA that does not encode all of the alphavirus structural proteins, purified glycoproteins E1 and E2, one or more non-cationic lipids, such as lecithin, and detergent. Detergent is slowly removed from the mixture to allow formation of lipid bilayers with incorporated RNA and glycoproteins.

25 In preferred embodiments of the methods of this invention, the glycoproteins E1 and E2 could be expressed in any recombinant protein expression system capable of glycosylation of mammalian proteins, such as stably transformed cell lines, for example CHO cells, or viral vector expression systems such as vaccinia, baculovirus, herpes virus, alphavirus or adenovirus. In a preferred embodiment, following expression of 30 the proteins; the E1 and E2 glycoproteins are purified from contaminating cellular

proteins in the expression supernatant. The purification of these glycoproteins can be achieved by affinity chromatographic column purification, for example using lectin-, heparin-, or antibody-affinity columns. This affinity purification step may be preceded by selective precipitation or selective extraction from the expression system supernatant

5 by methods including, but not limited to, ammonium sulfate precipitation or detergent extraction respectively. Final polishing steps of purification may include ion-exchange chromatography or buffer exchange, for example, and tangential flow methods to generate purified glycoproteins suitable for virosome assembly.

10 Thus, the present invention provides a method of producing alphavirus replicon virosomes, comprising: a) combining alphavirus replicon RNA, alphavirus glycoproteins E1 and E2, non-cationic lipids and detergent; and b) gradually removing detergent, whereby alphavirus replicon virosomes are produced. This method is described in more detail in the Examples section herein.

15 The present invention also provides alphavirus replicon virosomes comprising an alphavirus replicon RNA encapsidated by a lipid bilayer in which alphavirus glycoproteins are embedded. The replicon RNA can be from any alphavirus and the glycoproteins can be from any alphavirus. In a specific embodiment, the alphavirus 20 glycoproteins are VEE E1 and E2. The advantage of the alphavirus replicon virosomes is the ease of preparation, their stability, and their purity, since they are devoid of any cellular components being made in a cell free system.

25 The helper cells, RNAs and methods of the present invention are useful in *in vitro* expression systems, wherein the inserted heterologous RNA located on the replicon RNA encodes a protein or peptide which is desirably produced *in vitro*. The helper cells, RNAs, methods, compositions and pharmaceutical formulations of the present invention are additionally useful in a method of administering a protein or peptide to a subject in need of the desired protein or peptide, as a method of treatment 30 or otherwise.

It is contemplated that the nucleic acids, vectors and alphavirus replicon particles of this invention can be administered to a subject to impart a therapeutic or beneficial effect. Therefore, the nucleic acids, vectors and particles of this invention can be present in a pharmaceutically acceptable carrier. By "pharmaceutically acceptable" is meant a material that is not biologically or otherwise undesirable, i.e., the material may be administered to a subject, along with the nucleic acid or vector of this invention, without causing any undesirable biological effects or interacting in a deleterious manner with any of the other components of the pharmaceutical composition in which it is contained. The carrier would naturally be selected to minimize any degradation of the active ingredient and to minimize any adverse side effects in the subject, as would be well known to one of skill in the art (see, e.g., *Remington's Pharmaceutical Science*; latest edition).

Pharmaceutical formulations of this invention, such as vaccines, of the present invention can comprise an immunogenic amount of the alphavirus replicon particles as disclosed herein in combination with a pharmaceutically acceptable carrier. An "immunogenic amount" is an amount of the infectious alphavirus particles which is sufficient to evoke an immune response (humoral and/or cellular immune response) in the subject to which the pharmaceutical formulation is administered. An amount of from about 10^3 to about 10^7 replicon-containing particles, and preferably, about 10^4 to about 10^6 replicon-containing particles per dose is believed suitable, depending upon the age and species of the subject being treated. Exemplary pharmaceutically acceptable carriers include, but are not limited to, sterile pyrogen-free water and sterile pyrogen-free physiological saline solution.

25

Subjects which may be administered immunogenic amounts of the infectious, replication defective alphavirus particles of the present invention include, but are not limited to, human and animal (e.g., horse, donkey, mouse, hamster, monkey) subjects. Administration may be by any suitable means, such as intraperitoneal or intramuscular injection.

Pharmaceutical formulations for the present invention can include those suitable for parenteral (e.g., subcutaneous, intradermal, intramuscular, intravenous and intraarticular) administration. Alternatively, pharmaceutical formulations of the present invention may be suitable for administration to the mucous membranes of a subject (e.g., intranasal administration). The formulations may be conveniently prepared in unit dosage form and may be prepared by any of the methods well known in the art.

Thus, the present invention provides a method for delivering nucleic acids and vectors (e.g., alphavirus replicon particles; virosomes) encoding the antigens of this invention to a cell, comprising administering the nucleic acids or vectors to a cell under conditions whereby the nucleic acids are expressed, thereby delivering the antigens of this invention to the cell. The nucleic acids can be delivered as naked DNA or in a vector (which can be a viral vector) or other delivery vehicles and can be delivered to cells *in vivo* and/or *ex vivo* by a variety of mechanisms well known in the art (e.g., uptake of naked DNA, viral infection, liposome fusion, endocytosis and the like). The cell can be any cell which can take up and express exogenous nucleic acids.

Further provided herein is a method of inducing an immune response to an HIV antigen of this invention in a subject, comprising administering to the subject an immunogenic amount of the particles, virosomes and/or composition of this invention, in a pharmaceutically acceptable carrier.

A method of treating and/or preventing infection by HIV in a subject is also provided herein, comprising administering to the subject an effective amount of the particles, virosomes and/or compositions of this invention, in a pharmaceutically acceptable carrier.

The subject of this invention can be any animal in which an immune response can be induced or in which an infection by HIV can be treated and/or prevented. In a preferred embodiment, the subject of this invention is a mammal and most preferably is

a human.

Protocols and data regarding the testing of the compositions of this invention in animals and protocols for administration to humans are provided in the Examples 5 herein.

In a particular embodiment, the present invention provides an isolated nucleic acid encoding a *pol* gene product or immunogenic fragment thereof of a human immunodeficiency virus, wherein the integrase, RNase H and reverse transcriptase 10 functions of the *pol* gene product or immunogenic fragment thereof have been inactivated or deleted. Such a modification has been shown in some studies to facilitate inhibition of the formation of replication competent alphavirus particles during production of alphavirus replicon particles comprising the *pol* gene product or immunogenic fragment thereof.

15

Also provided herein is a composition comprising the *pol*-expressing nucleic acid described above, a vector comprising the nucleic acid and a cell comprising the vector. The *pol*-expressing nucleic acid can also be present in an alphavirus replicon particle comprising the nucleic acid.

20

As noted above, the nucleic acid encoding the *pol* gene product or immunogenic fragment thereof comprises a modification resulting in the inhibition of reverse transcriptase activity. In a preferred embodiment, a mutation is introduced at the active site motif that results in inhibition of reverse transcriptase activity. Such a mutation 25 may remove the DNA binding domain of the enzyme, for example. A mutation from YMDD to YMAA or HMAA at this motif is an example of such a mutation.

The present invention additionally provides a method of making an alphavirus replicon particle comprising nucleic acid encoding a *pol* gene product or immunogenic 30 fragment thereof of a human immunodeficiency virus, wherein the *pol* gene product or

immunogenic fragment thereof comprises a modification resulting in deletion or inactivation of integrase, RNase H and reverse transcriptase functions from the *pol* gene product or immunogenic fragment thereof, comprising

5 A) providing a helper cell for producing an infectious, defective alphavirus particle, comprising in an alphavirus-permissive cell:

(i) an alphavirus replicon RNA, wherein the replicon RNA comprises an alphavirus packaging signal and a nucleic acid encoding a *pol* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, wherein the *pol* gene product or immunogenic fragment thereof is modified to delete or inactivate RNase H, integrase and reverse transcriptase functions in the *pol* gene product or immunogenic fragment thereof, and wherein the replicon RNA lacks sequences encoding alphavirus structural proteins;

10 (ii) a first helper RNA separate from said replicon RNA, said first helper RNA encoding at least one alphavirus structural protein and furthermore not encoding at least one other alphavirus structural protein; and

15 (iii) one or more additional helper RNA(s) separate from said replicon RNA and separate from said first helper RNA, said additional helper RNA(s) encoding at least one other alphavirus structural protein not encoded by said first helper RNA;

20 and with at least one of said helper RNAs lacking an alphavirus packaging signal;

25 wherein the combined expression of the alphavirus replicon RNA and the helper RNAs produces an assembled alphavirus particle which is able to infect a cell, and is unable to complete viral replication, and further wherein the population contains no detectable replication-competent alphavirus particles as determined by passage on permissive cells in culture;

30 (B) producing the alphavirus particles in the helper cell; and

(C) collecting the alphavirus particles from the helper cell.

In the method provided above, at least one of the replicon RNA, the first helper RNA, and the one or more additional helper RNA(s) can comprise one or more attenuating mutations, as described herein.

5 In a specific embodiment of this method, a mutation is introduced at the active site motif in the *pol* gene product or immunogenic fragment thereof that results in inhibition of reverse transcriptase activity. Such a mutation may remove the DNA binding domain of the enzyme, for example. A mutation from YMDD to YM_{AA} or H_{MAA} at this motif is an example of such a mutation.

10

Also provided herein is an alphavirus replicon particle expressing the *pol* gene product or immunogenic fragment thereof, wherein the *pol* gene product or immunogenic fragment thereof comprises a modification resulting in deletion or inactivation of integrase, RNase H and reverse transcriptase functions in the *pol* gene product or immunogenic fragment thereof, produced according to any of the above methods.

15 In a further embodiment, the present invention provides a method of inducing an immune response in a subject, comprising administering to the subject an immunogenic amount of a composition comprising an alphavirus particle comprising nucleic acid encoding a *pol* gene product or immunogenic fragment thereof of a human immunodeficiency virus, wherein the *pol* gene product or immunogenic fragment thereof comprises a modification resulting in deletion or inactivation of integrase, RNase H and reverse transcriptase functions in the *pol* gene product or immunogenic fragment thereof, in a pharmaceutically acceptable carrier.

20 Furthermore, the present invention provides a method of treating or preventing infection by human immunodeficiency virus in a subject, comprising administering to the subject an effective amount of a composition comprising an alphavirus particle comprising nucleic acid encoding a *pol* gene product or immunogenic fragment thereof

of a human immunodeficiency virus, wherein the *pol* gene product or immunogenic fragment thereof comprises a modification resulting in deletion or inactivation of integrase, RNase H and reverse transcriptase functions in the *pol* gene product or immunogenic fragment thereof, in a pharmaceutically acceptable carrier.

5

In preferred embodiments of the methods of this invention, the subject is administered an effective amount of a population of alphavirus particles comprising particles expressing (1) nucleic acid encoding a *pol* gene product or immunogenic fragment thereof of a human immunodeficiency virus, wherein the *pol* gene product or immunogenic fragment thereof comprises a modification resulting in inactivation or deletion of integrase, RNase H and reverse transcriptase functions in the *pol* gene product or immunogenic fragment thereof, (2) nucleic acid encoding a *gag* gene product or immunogenic fragment thereof of a human immunodeficiency virus, wherein the *gag* gene product or immunogenic fragment thereof is modified to inhibit release of *gag* gene product or the immunogenic fragment thereof from a cell, and (3) nucleic acid encoding an *env* gene product or an immunogenic fragment thereof of a human immunodeficiency virus in a pharmaceutically acceptable carrier.

In further preferred embodiments, the population of alphavirus particles comprises particles expressing (1) nucleic acid encoding a *gag* gene sequence that has at least 92% identity with SEQ ID NO:4; (2) nucleic acid encoding a *pol* gene sequence that has at least 99% identity with SEQ ID NO:15; and (3) nucleic acid encoding an *env* gene sequence with at least 95% identity with SEQ ID NO:18. In a specific embodiment, the population of alphavirus particles comprises particles expressing (1) nucleic acid of SEQ ID NO:4, (2) nucleic acid of SEQ ID NO:15, and (3) nucleic acid of SEQ ID NO:18.

EXAMPLES

30 The following examples are provided to illustrate the present invention, and

should not be construed as limiting thereof. In these examples, nm means nanometer, mL means milliliter, pfu/mL means plaque forming units/milliliter, VEE means Venezuelan Equine Encephalitis virus, EMC means encephalomyocarditis virus, BHK means baby hamster kidney cells, HA means hemagglutinin gene, N means

5 nucleocapsid, FACS means fluorescence activated cell sorter, and IRES means internal ribosome entry site. The expression "E2 amino acid (e.g., lys, thr, etc.) number" indicates the designated amino acid at the designated residue of the E2 gene, and is also used to refer to amino acids at specific residues in the E1 protein and in the E3 protein, respectively.

10

EXAMPLE 1

VEE Replicon Particles as Vaccines

Replicon particles for use as a vaccine are produced using the VEE-based vector system, originally developed from a full-length, infectious cDNA clone of the RNA genome of VEE (Figure 1 in Davis *et al.*, 1989). In this Example, one or more attenuating mutations (Johnston and Smith, 1988; Davis *et al.*, 1990) have been inserted into the clone to generate attenuated VEE vaccine vectors (Davis *et al.*, 1991; 1995; Grieder *et al.*, 1995).

20

As described herein, these constructs are genetically modified to create an RNA replicon (i.e., an RNA that self-amplifies and expresses), and one or more helper RNAs to allow packaging. The replicon RNA expresses an HIV gene, e.g., the Clade C HIV-1 *gag* gene. The replicon RNA is packaged into virus-like particles (herein referred to 25 as "virus replicon particles" or "VRPs") that are infectious for only one cycle. During this cycle, the characteristics of the alphavirus-based vector result in very high levels of expression of the replicon RNA in cells to which the VRP is targeted, e.g., cells of the lymph node.

30

In the cytoplasm of the target cell, the replicon RNA is first translated to

produce the viral replicase proteins necessary to initiate self-amplification and expression. In this Example, the HIV-1 Clade C *gag* gene is encoded by a subgenomic mRNA, abundantly transcribed from a negative-sense replicon RNA intermediate, leading to high-level expression of the HIV-1 Clade C *gag* gene product. Since the 5 VEE structural protein genes are not encoded by the replicon RNA, progeny virion particles are not assembled, thus limiting the replication to a single cycle within the infected target cell.

Importantly, only the replicon RNA is packaged into VRPs, as the helper RNAs 10 lack the *cis*-acting packaging sequence required for encapsidation. The "split helper" or bipartite system (see Example 4) greatly reduces the chance for an intact genome being assembled by recombination, and as a back-up safety feature, one or more highly attenuating mutations, such as those contained in the glycoprotein genes in V3014 (Grieder *et al.*, 1995), are incorporated.

15

Overall, the design of the VRPs incorporates several layered and redundant safety features. In addition to the above-described split helper system and attenuating mutations, over one-third of the genome of the virus has been removed, creating a defective genome which prevents spread from the initially infected target cell. 20 Nonetheless, if a statistically rare recombination event occurs to yield replication competent virus (RCV), the resulting virus would be a highly attenuated VEE strain.

EXAMPLE 2

Construction of VEE Replicon

25

The VEE structural protein genes (C-PE2-6K-E1) are removed from a cDNA clone (pV4031) which contained two attenuating mutations (E2 lys 209, E1 thr 272), and a duplication of the 26S subgenomic RNA promoter sequence immediately downstream from the 3'-end of the E1 glycoprotein gene, followed by a multiple 30 cloning site as described in U.S. Pat. No. 5,505,947 to Johnston *et al.* The pV4031

plasmid DNA is digested to completion with ApaI restriction enzyme, which cuts the VEE genomic sequence at nucleotide 7505 (numbered from the 5'-end of the genome sequence). A second recognition site for this enzyme is found in the duplicate 26S subgenomic promoter. Therefore, digestion of pV4031 with ApaI produces two DNA

5 fragments, one containing the VEE nonstructural genes (e.g. SEQ ID NO:2) and a single copy of the 26S subgenomic RNA promoter followed by a multiple cloning site, and a second smaller fragment containing a 26S subgenomic RNA promoter followed by the VEE structural genes. The large fragment is isolated and religated to produce the replicon, pVR2. In this example, as well as in the construction of the helper plasmids

10 (Example 3), a kanamycin resistance gene (SEQ ID NO:6, encoding amino acid sequence as in SEQ ID NO:7) is present in the plasmids to aid in the cloning manipulations.

EXAMPLE 3

15 Construction of Helper Plasmids

The starting materials for the helper plasmids are four full-length cDNA clones: V3000, the virulent Trinidad donkey strain of VEE, three clones with attenuating mutations, pV3014 (E2 lys 209, E1 thr 272), V3519 (E2 lys 76, E2 lys 209, E1 thr 272)

20 and V3526 (deletion of E3 56-59, E1 ser 253), which are in the genetic background of Trinidad donkey strain VEE. Several different helper plasmids have been made by using unique or rare restriction sites in the full-length cDNA clone to delete portions of the nonstructural protein region. The full-length clone is digested with one or two restriction enzymes, the larger DNA fragment is isolated and then religated to form a

25 functional plasmid. *In vitro* RNA transcripts from these plasmids upon transfection of tissue culture cells would not encode a functional RNA replication complex, and also would not include an encapsidation signal. The helper constructs differ in the size of the nonstructural gene deletion. The helper constructs are designated by the attenuated mutant clone used in their construction, and by the percentage of the nonstructural

30 region deleted. The following helper constructs were generated:

V3014Δ520-7507(93%)
V3519Δ520-7507(93%)
V3526Δ520-7505(93%)
V3014Δ520-6965(87%)
5 V3519Δ1687-7507(78%)
V3014Δ2311-7505(70%)
V3519Δ3958-7507(47%)
V3526Δ520-7505(93%)
V3014Δ3958-7505(47%)
10 V3519Δ1955-3359(19%)
V3014Δ520-3954(46%)
V3014Δ1955-3359(19%)
V3014Δ1951-3359(19%)
V3014Δ2311-3055(10%)
15 V3014Δ2307-3055(10%)

EXAMPLE 4
Construction of Bipartite RNA Helper Plasmids

20 A bipartite helper system is constructed as described herein. The V3014Δ520-7505(93%) helper is used to construct an additional deletion of the E2 and E1 glycoprotein genes by digestion with HpaI restriction enzyme and ligation, resulting in deletion of the sequence between nucleotide 8494 (in the E3 gene) and nucleotide 11,299 (near the 3'-end of the E1 gene). *In vitro* RNA transcripts of this glycoprotein 25 helper plasmid (presented graphically in Figure 2; an exemplary nucleotide sequence for such a plasmid is SEQ ID NO:8, including the nucleotide sequence (SEQ ID NO:9 and the amino acid sequence (SEQ ID NO:10 of the VEE capsid), when electroporated into BHK cells with a replicon RNA, are replicated and transcribed to give a mRNA encoding only the capsid protein of VEE.

The second member of the bipartite helper is constructed from the same original helper plasmid 3014Δ5207505(93%) by cleavage with Tth11I restriction enzyme (at nucleotide 7544) and SpeI restriction enzyme (at nucleotide 8389), resulting in deletion of the capsid gene, followed by insertion of a synthetic double-stranded oligonucleotide 5 with Tth11I and SpeI termini. The inserted sequence restored the downstream portion of the 26S promoter and an ATG initiation codon followed by a Ser codon, such that the first amino acid residue of E3 (Ser) is the first codon following the inserted AUG. The resulting glycoprotein helper plasmid is presented graphically in Figure 3, and an exemplary nucleic acid sequence for such a plasmid is SEQ ID NO:11, encoding the 10 VEE glycoproteins (E3-E2-6kD-E1), SEQ ID NO:12. The *in vitro* transcript of this plasmid, when transfected into a cell with replicon RNA, will produce the VEE glycoproteins (SEQ ID NO:13). Co-electroporation of both of these helper RNAs into a cell with replicon RNA results in production of infectious particles containing only replicon RNA.

15

Other than the 5' and 3' ends and the 26S promoters (40 nucleotides) of these helper RNAs, the only sequence in common between the capsid and glycoprotein helpers is the sequence from 8389 to 8494 (106 nucleotides)

20

EXAMPLE 5

VEE REPLICON PARTICLES EXPRESSING HIV GENES

The vaccines of this invention are exemplified by the use of a propagation defective, replicon particle vector system derived from an attenuated strain of 25 Venezuelan equine encephalitis virus (VEE) to create a mixture of VEE replicon particles individually expressing HIV-1 *gag*, *pol*, or *env* genes. The three genes used in this Example were selected based on homology to consensus sequences generated from primary isolates obtained from recent seroconverters in Kwazulu/Natal. Plasma samples from approximately 20 recent seroconverters in the Durban/Hlabisa cohort and 30 a similar number of HIV-positive, asymptomatic individuals were collected. HIV viral

RNA was isolated from the plasma, and the sequences of the *gag*, *pol* and *env* genes were analyzed. Two regions from each gene were amplified, and the resulting PCR products were sequenced (see Figure 10 for regions analyzed). A consensus sequence was derived for each gene, and the sequences of each isolate were compared to the 5 derived consensus. All isolates were found to be Subtype C of HIV, thus confirming the predominance of this subtype in South Africa.

A. CONSTRUCTION OF THE Gag-VRP VACCINE

10 Described herein is the design and manufacture of VEE replicon particles (VRPs) engineered to express the *gag* gene from a Subtype C isolate of HIV-1. The main purpose of this single antigen vaccine is to establish a safety profile for VRPs in healthy human subjects. Optimally, the HIV-Gag-VRPs will be formulated as a component of a trivalent vaccine, also containing HIV-Pol-VRP and HIV-gp160-VRP 15 (*env*) made in analogous procedures to the one described herein for HIV-Gag-VRPs.

In this Example, the VEE particles are based on the V3014 glycoprotein helper plasmid (Figure 3, SEQ ID NO:12 and SEQ ID NO:13), which harbors two highly attenuating mutations, one in E2 and the other in E1 (Grieder *et al.*, 1995). The V3014 20 glycoprotein helper RNA is able to package VRPs with significantly greater efficiency than the glycoprotein helper RNA derived from V3526 (Pushko *et al.*, 1997). Nonetheless, safety of the VRP vector system has not been compromised since detailed pathogenesis studies clearly have shown V3014 to be avirulent in adult mice by 25 subcutaneous inoculation (Grieder *et al.*, 1995). V3014 was found to be significantly impaired in its ability to reach and spread beyond the draining lymph node following subcutaneous inoculation. Unlike wild-type V3000, V3014 does not establish a viremia and does not reach the brain. In addition, on rare occasions when found, histopathological lesions in the periphery were much less severe than those induced by wild-type V3000 (Grieder *et al.*, 1995). Following inoculation with V3014, adult mice 30 are protected against lethal wild-type VEE infection.

The attenuated phenotype of V3014 also was observed in VEE challenge studies in horses. Animals inoculated subcutaneously with V3014 showed no significant leukopenia or febrile response compared to mock-vaccinated controls. In addition, results indicated that these animals were completely protected against virulent 5 VEE (V3000) challenge.

Taken together, these data indicate that if the rare recombination event did occur during VRP assembly to yield RCV, the worst case scenario would be the generation of a highly attenuated strain of VEE.

10

B. SELECTION AND CLONING OF THE HETEROLOGOUS ANTIGEN

The exemplary HIV genes used in this invention, *gag*, *pol* and *env*, are derived from Subtype C (Clade C) viruses isolated from likely Phase III clinical trial sites in 15 South Africa. The HIV infection rate in South Africa and its long established virology and public health infrastructure make this country an attractive choice for clinical testing of HIV vaccines. Focused sequencing and phylogenetic analysis of the *gag*, *pol*, and *env* genes of these isolates has allowed the selection of genes representative of the Clade C isolates circulating in this region of Africa.

20

1. HIV-1 Clade C *gag* gene

Two 400 bp regions of the *gag* gene were sequenced from approximately 30 plasma samples collected from HIV seropositive individuals in South Africa. A South African consensus sequence was then determined for the *gag* gene as well as a 25 consensus sequence from the Los Alamos database for Subtype C virus. In addition, approximately 20 comparable sequences from Malawi were used, generated as part of another study, to confirm conclusions about sequence variation. Several isolates that were close to the South African consensus sequence were compared to other isolates in distance measurements. Among these 30 isolates, one was chosen as the source for the 30 *gag* gene (SEQ ID NO:4; corresponding to the amino acid sequence in SEQ ID NO:5)

for the following reasons.

This isolate had greater than 95% amino acid identity to the South African consensus sequence, representing the approximate middle of the sequence diversity of 5 all isolates. This isolate, known as DU422, came from a recent seroconvertor, reflecting currently circulating strains and the transmitted phenotype. The phenotype of DU422 is NS1, CCR5(+), and CXCR4(-).

Prior to the insertion of the *gag* gene into the VEE replicon plasmid vector, the 10 amino terminal myristylation ("myr") site of *gag* was removed to prevent the formation of Gag-containing virus-like particles. Restriction enzyme digests of the *gag* gene plasmid, the capsid helper plasmid, and the glycoprotein helper plasmid were performed to confirm the identity of the three vectors when compared to published 15 maps of the parental plasmid pBR322, with the kanamycin resistance gene substituted for the ampicillin resistance gene. The confirmed plasmid maps of the VEE replicon plasmid containing the DU422 *gag* gene (p3-40.1.6), the capsid helper plasmid (p3-13.2.2), and the glycoprotein helper plasmid (p3-13.4.6) are presented in Figures 1, 2, and 3, respectively. The full nucleotide sequence of each of these plasmids is presented herein as SEQ ID NO:1, SEQ ID NO:8, and SEQ ID NO:11, respectively.

20

In Figures 6 and 15, expression of this HIV-1 Gag protein in BHK cells infected with VRPs expressing such a *gag* construct is demonstrated (Figure 6: Western blot, lane 3; Figure 15, immunofluorescence detection). The cells were infected at a 25 multiplicity of infection (m.o.i.) of 3.5 infectious units (i.u.) per cell, and expression was measured 18 hours post-infection (p.i.). Cell lysates (from approximately 2×10^3 cells) were collected and fractionated either by a 4-12% gradient SDS-PAGE or by 10% SDS-PAGE. The fractionated polypeptides were transferred to PVDF membranes and probed with human HIV-1 positive serum.

30

1. HIV-1 Clade C *env* gene

A Clade C *env* gene (aka "gp160") from another HIV isolate, DU151, from a recent seroconverter was chosen based on its 92% amino acid identity to the South African consensus sequence for this gene, determined in an analogous method to the one described for the *gag* gene in Example 5.A.1. The phenotype of the DU151 isolate is NS1, CCR5(+), CXCR4(-). This gene was engineered into a VEE RNA replicon plasmid as shown in Figure 5, and the entire sequence of the plasmid is given at SEQ ID NO:17. The *env* gene construct used in this Example is SEQ ID NO:18.

In Figure 6, expression of this ENV protein (SEQ. ID. NO:19) in BHK cells infected with VRPs expressing this HIV *env* construct is demonstrated (Western blot, lane 2), showing that the protein expressed in the cells is of the correct size and is immunoreactive. In Figure 7, expression of this ENV protein in U87.CD4.CCR5 cells is shown. These cells process the ENV protein into two components, GP120 and GP41. In these cells, the expressed GP160 is fusogenic (see Figure 8).

3. HIV-1 Clade C *pol* gene

A Clade C *pol* gene from isolate DU151 was chosen based on its 99% amino acid identity with the South African consensus sequence. This gene was modified at the active site of the reverse transcriptase encoding sequence to inhibit its activity, and the p51 fragment of this modified gene (SEQ ID NO:15) was engineered into a VEE RNA replicon plasmid. The map of this *pol* plasmid is shown in Figure 4, and the nucleotide sequence of the plasmid is provided as SEQ ID NO:14. In Figure 6, expression of this POL p51 fragment (SEQ ID NO:16) in BHK cells is demonstrated (Western blot, lane 1), showing that the protein expressed in these cells is both the correct size and immunoreactive.

C. IMMUNOLOGICAL RESPONSE TO VRP-GAG VACCINE

Mice were injected subcutaneously in two doses, with 8-9 mice in each group. The mice were immunized once, then immunized a second time, with the same dose, 28 5 days later. Serum was collected the day prior to the first immunization, then at day 27 ("after 1st immunization) and at day 35 (after 2nd immunization).

The vigorous, antigen-specific humoral response of mice to the HIV-1 Clade C VRP-gag vaccine described in Example 5.A.1. is presented in Table 1. Details of this 10 assay are described in Example 7A.1.

TABLE 1. Humoral Response to VRP-gag Vaccine

		Total Ab Titer (log ₁₀)
15	Dose:	
	10 ³ i.u. dose:	
	after 1 st immunization	1.3 +/- 0.1
	after 2 nd immunization	2.8 +/- 1.1
20	10 ⁵ i.u. dose	
	after 1 st immunization	2.1 +/- 0.5
	after 2 nd immunization	4.1 +/- 0.6

The vigorous, antigen-specific CTL response in mice to the HIV-1 Clade C 25 VRP-gag vaccine (Example 5.A.1) is presented in Figure 9. Details of this assay are described in Example 7A.3.

EXAMPLE 6
MANUFACTURING PROCESS FOR HIV VRP VACCINES

A. Manufacturing Process

5

Disclosed herein is a manufacturing process for VRP vaccines that is suitable for large-scale preparation of GMP-compliant (GMP = Good Manufacturing Practices) material for use in human clinical trials or for commercial manufacture. The process includes several steps and after each step (as appropriate), a set of "in process control" 10 (IPC) assays or Release Tests (RT) is performed to confirm the successful completion of the step. The process steps and the accompanying IPC assay(s) or RTs (described in more detail in Example 6D.1 and 6D.2) are as follows:

	Process Step	IPC/RT
15	Linearize 3 DNA plasmids	IPC: Check for linearity
	<i>In vitro</i> RNA transcription	IPC: Size, integrity and concentration
	Electroporation of certified Vero cell line	IPC:
	Harvest culture fluids	Titration/Identity
		Test for replication-competent virus
		(RCV)
	Pool the culture fluid	RT:
		Mycoplasma
		Adventitious virus
		PERT assay
20	Purification of bulk VRP by heparin affinity chromatography	IPCs:
		Heparin residual assay
		BSA assay
		Bovine IgG assay

Filtration of bulk VRP

RT:

Test for RCV

Titration/Identity

Contaminating protein/DNA

Sterility

Endotoxin

RT:

Titration/Identity

Sterility

General Safety

Formulate, Fill, Release

B. Preparation of plasmid DNAs

5 Stock solutions of replicon plasmid DNA, capsid helper plasmid DNA and glycoprotein helper plasmid DNA are produced in *Eschericia coli* XL2 Blue cells (Stratagene, cat# 200150). All plasmids harbor the kanamycin resistance gene marker. The three plasmid DNAs were manufactured and purified by PureSyn, Inc. (Malvern, PA) under appropriate GLP/GMP procedures, with a complete Batch Record with full
10 traceability. Following fermentation and cell harvest, cell paste was lysed with base and plasmid DNAs were purified by ion pair chromatography on PolyFlo™ separation media.

Prior to release by appropriate quality assurance/quality control oversight, each
15 lot of each plasmid DNA is analyzed to confirm identity, purity and quality (Table 2). An approved certificate of analysis for each DNA is then established for each plasmid DNA lot.

Table 2. Plasmid DNA Release Tests

Test	Method	Specification
DNA homogeneity	Agarose gel electrophoresis	>90% supercoiled
<i>E. coli</i> genomic DNA	Southern Blot	<50 µg/mg plasmid
<i>E. coli</i> RNA	Agarose gel electrophoresis	No detectable bands
Endotoxin	Limulus Amoebocyte Lysate (LAL)	< 0.1 EU/mg
Total protein	Abs 260/280	1.8-1.9
Sterility	Bioburden assay, USP23	< 1 CFU
Identity	Restriction enzyme analysis	Matches map

20 To produce HIV-VRP vaccine for clinical use, both replicon and helper plasmids are linearized by digestion at the unique Not I site and used as templates for synthesis of run-off transcripts. The quality of the transcription products (i.e., the replicon and the two helper RNAs) is evaluated by agarose gel electrophoresis.

25 **C. Characterization of the Vero cells**

Vero cells are used in the production of HIV-VRPs (WHO Vero MCB P139, BioReliance Inc., Rockville, MD). Vials contained approximately 1×10^7 cells/mL in a cryoprotectant solution of 90% fetal bovine serum and 10% dimethyl sulfoxide. A Cell 30 Certification Summary is provided with each lot. BioReliance Inc. has filed a Master File with the FDA regarding the WHO Vero MCB P139.

Vials of WHO Vero MCB P139 cells are expanded into flasks. Each of the flasks is then expanded again in order to prepare the Master Cell Bank (MCB). The Working Cell Bank (WCB) is prepared from the MCB. The MCB is tested for purity and identity. The WCB is tested for adventitious agents (detection of mycoplasma and viruses).
5 Viability tests are performed on both the MCB and the WCB.

Tumorigenicity tests are performed once at the end of the production period.

D. Electroporation

10

Vero cells are cotransfected by electroporation with RNA mixtures comprising replicon RNA transcripts encoding HIV-gag, VEE capsid helper RNA transcripts, and VEE glycoprotein helper RNA transcripts. The transfected cells are transferred to tissue culture vessels and incubated in well-defined culture medium. Following harvest, the
15 HIV-Gag-VRP is purified from pooled culture fluid supernatants by affinity column chromatography. Prior to formulation and filling, purified, bulk HIV-Gag-VRP is tested for the presence of RCV.

E. Final formulated product

20

The HIV-Gag-VRP vaccine is vialled at four different doses. The material is filtered (0.22 µm) and added to vials at the appropriate concentration and volume, stoppered, quick-frozen and stored at -20° C.

25 **F. Control tests of the Gag-VRP vaccine**

1. In-Process Controls

Table 3 below summarizes the In-Process Controls performed during the
30 manufacturing process of the HIV-Gag-VRP Vaccine.

Table 3. IPCs during the manufacture of HIV-Gag-VRP Vaccine

	Test	Method	Target
5	Check for linearity	Agarose Gel electrophoresis	Report
10	Size, integrity and concentration of RNAs	Agarose Gel electrophoresis	Report
15	Titration/Identity	Indirect immunofluorescence assay(IFIA), using standardized Gag-specific antibody preparation	Report
20	Test for RCV	CPE Assay	Report
	Heparin Residual	Chromogenic Inhibitory Assay	Report
	BSA residual	ELISA	Report
	Bovine IgG Residual	ELISA	Report

2. Release tests

Tables 4 and 5 below summarize the release tests performed on the HIV-Gag-VRP Vaccine.

Table 4. Pool of the Culture Fluids

Test	Method	Target
Adventitious Virus (<i>in vivo</i>)	European guidelines	Negative
Adventitious Virus (<i>in vitro</i>)	5 cell lines	No growth
Mycoplasma	21CFR 610.30	No Growth
Reverse Transcriptase	PERT Assay	Negative

Table 5. Bulk VRP and Final Vial testing

Test	Method	Target Result
Replication competent virus (RCV)	Cytopathic effect (CPE) assay	Absence (in BHK cells, sensitivity is 1-10 pfu V3014)
VRP identity/ potency	Indirect immunofluorescence assay (IFA)	10^6 to 10^8 i.u. per mL
Cellular Protein Contaminant	Bio-Rad® DC protein assay	Total protein content per dose
Cellular DNA Contaminant	Southern Blot or PCR	< 10 ng per dose
Sterility	21 CFR § 610.12	Pass

Endotoxin	LAL	< 5 EU/dose
General Safety	21 CFR § 610.11	Pass
Particulates	USP	Pass
Stability	IFA	10^6 to 10^8 i.u. per mL

10

EXAMPLE 7
PRECLINICAL STUDIES

Pilot lots are manufactured following written procedures (SOPs and STMs) and according to the manufacturing scheme described in Example 6. These pilot lots are 15 prepared and used for two major tasks. The first one is a preclinical immunogenicity evaluation, which includes studies to assess the immune response and the cell-mediated immune response in vaccinated animals. The second major task is a preclinical safety evaluation, which includes evaluations of system toxicity, hematopoietic and immune system toxicity, and local reactogenicity.

20

Finally, an *in situ* hybridization study is performed in mice in order to verify the *in vivo* expression of HIV-Gag-VRP gene product in lymphoid tissue.

A. Immunogenicity Studies

25

A.1 Humoral Immune Response in Mice

Three groups of five female BALB/c mice (4-6 weeks of age) are inoculated subcutaneously with 10^5 , 10^6 , or 10^7 i.u. of the HIV-Gag-VRP at three time points: on 30 day 0, and at weeks 4 and 8. The fourth group, Control Group, receives the vehicle only. Immediately prior to inoculation, and at weeks 3, 5, 8 and 10 post-inoculation,

blood samples are collected for humoral immune response evaluations. Gag protein-specific serum antibody titers and seroconversion rates are measured by ELISA (Caley *et al.*, 1997) against purified, recombinant Gag protein. The source of the antigen is the homologous Clade C *gag* gene expressed in insect or mammalian cells. Antigen specificity also is confirmed by immunoblot analysis. Anti-VEE responses are monitored by ELISA (Johnston and Smith, 1988).

A.2 Humoral Immune Response in Rabbits

10 Three groups of five female New Zealand white rabbits are inoculated subcutaneously with 10^5 , 10^6 , or 10^7 i.u. of the HIV-Gag-VRP. The fourth group, Control Group, receives the vehicle only. Immediately prior to inoculation, and at weeks 3, 5, 8 and 10 post-inoculation, blood samples are collected for humoral immune response evaluations.

15 Humoral immune responses are evaluated as described in Section A.1.

A.3 Cell-Mediated Immune Response in Mice

20 Three groups of five female BALB/c mice are inoculated subcutaneously with 10^5 , 10^6 , or 10^7 i.u. of the HIV-Gag-VRP at day 0 and day 28. The fourth group, Control Group, receives the vehicle only. Blood samples are collected at week 3 post-inoculation. Spleens are harvested for splenocyte collection on day 7 following the second inoculation for evaluation of cell-mediated immune responses.

25 The cell-mediated immune response is evaluated by determining the ability of splenic T cells from immunized mice to proliferate *ex vivo* in the presence of either Gag protein or Gag peptide(s). The ability of splenic T and CD4+ T cells to produce interferon- γ and interleukin-4 respectively, is determined. Finally, the ability of 30 cytotoxic T lymphocytes to lyse target cells that present murine major

histocompatibility complex class-I restricted epitopes for HIV-1 Clade C Gag protein is measured (see Betts *et al.*, 1997 for methods)

B. Safety Study

5

Three groups of six male and six female New Zealand white rabbits are inoculated subcutaneously with 10^4 , 10^6 , or 3×10^7 i.u. of the HIV-Gag-VRP. The fourth group, Control Group, receives the vehicle only. Animals receive four injections at week 0, week 3, week 6 and Week 9. Half of the animals are sacrificed two days 10 after the last injection (week 9) and the other half at three weeks after the last injection (week 12). Similar studies are performed in mice with a high dose at 10^8 i.u. This level is 100 times the likely primate dose, based on efficacy studies in rhesus macaques.

In addition to system toxicity (record of mortality/morbidity, body temperature, 15 body weight, food consumption and ophthalmic examinations), hematopoietic toxicity is evaluated by quantitating cellular components of peripheral blood, and immune system toxicity is assessed by histopathologic evaluation of the lymphoid organs. Local reactogenicity is evaluated by examining the injection sites grossly and microscopically to determine irritation potential. Serum samples are also tested for the 20 presence of replication competent virus by blind passage in cell culture.

C. *In Situ* Hybridization Study in Mice

Three groups of five female BALB/c mice are inoculated subcutaneously with 25 10^5 , 10^6 , or 10^7 i.u. of the HIV-Gag-VRP. The fourth group, Control Group, receives the vehicle only. A single injection is performed in each group.

To verify expression of HIV-GAG-VRP in lymphoid tissue, the draining lymph nodes, spleen, and thymus of the mice are examined by *in situ* hybridization at 24 hours 30 and 48 hours after the single inoculation.

EXAMPLE 8**Heparin Affinity Chromatography of VRPs**

5 Generally, the majority of contaminating protein is non-VEE protein from the conditioned media. Heparin column capacity requirements for GMP manufacturing runs are therefore based on the volume of conditioned media, rather than the concentration of VRPs. Column parameters are optimized at room temperature, but variations in temperature do not greatly affect performance. The expected yields of
10 VRPs can range from 50% to > 90%.

While only minimal leaching of heparin from the columns has been detected, GMP requirements stipulate that a residual heparin assay be performed as an IPC test following the chromatography step.

15

A. Pharmacia HiTrap® Heparin

Five mL columns of Pharmacia HiTrap® Heparin (cat no. 17-0407-01, Amersham Pharmacia Biotech), pre-equilibrated with 25 mM HEPES/0.25 M NaCl, pH
20 7.5, were loaded with HIV-Gag-VRPs produced in Vero cells. After column washing with the equilibration buffer, VRPs were eluted with a 15 column volume gradient from 0.25 – 1.0 M NaCl gradient in 25 mM HEPES, pH 7.5. The HIV-Gag-VRPs eluted at a conductivity of approximately 48 mS/cm. The wash step was optimized (based on the A₂₈₀ peak) at a NaCl concentration between 0.25 M and 0.3 M.

25

B. Heparin Sepharose 6 Fast Flow® resin

Heparin Sepharose 6 Fast Flow® resin (catalog no. 90-1000-2; Amersham Pharmacia Biotech) is supplied as a bulk resin which allows various size columns to be
30 packed as needed. Fast Flow® resins have the advantages of excellent flow

characteristics and ability to be sanitized with sodium hydroxide solutions, which are particularly useful in a GMP manufacturing process. A 6 mL column was prepared by packing the Heparin Sepharose 6 Fast Flow® bulk resin in a BioRad® Econo-Column chromatography column, which was then pre-equilibrated with 25 mM HEPES/0.12 M 5 NaCl, pH 7.5. VRPs were loaded onto the column, which was then washed with the equilibration buffer. Initial experiments indicated that the VRPs eluted at a lower conductivity (36 mS/cm) with this resin as compared to the HiTrap® Heparin, so the wash conditions were modified accordingly. The VRPs were eluted from the Fast Flow® resin with a 15 column volume gradient from 0.12 M to 1 M NaCl in 25 mM 10 HEPES, pH 7.5.

EXAMPLE 9

Virosome Formation

15

The feasibility of virosome formation is demonstrated in a series of experiments in which replicon RNA and RNA encoding the glycoprotein E1 and E2 genes (glycoprotein helper) were first transfected into BHK cells by electroporation. After 18-24 hours, cell supernatants were harvested and tested for the presence of virosomes 20 as described briefly below.

Cell Culture

BHK cells were used as a cell substrate and were maintained in growth medium 25 (alpha-MEM (Life Technologies), supplemented with 10% Fetal Bovine Serum (HyClone), 1x Glutamine (Life-Technologies)), in an atmosphere of 5% CO₂ at 37°C. Prior to electroporation, cells were detached from the cell culture vessel using 0.05% trypsin-0.53 mM EDTA solution (Life Technologies). Trypsin was neutralized with growth medium, and cells were washed twice with cold Phosphate-Buffered Saline 30 (PBS, BioWhittaker) and resuspended at a concentration of 1.5 x 10⁷ cells/ml.

RNA Transcription, Electroporation and Virosome Harvest

Plasmid DNA pVR-GFP (green fluorescent protein) was linearized using restriction endonuclease NotI (New England Biolabs) as recommended by the manufacturer. DNA was extracted with phenol:chloroform:iso-amyl alcohol (25:24:1, Gibco BRL) and precipitated with ethanol, following the addition of NH₄Ac to 2.5 M final concentration. RNA was synthesized in an *in vitro* transcription reaction using an mMessage mMachine® kit (Ambion) as recommended by the manufacturer. This RNA, without further purification, was used to transfect BHK cells. Helper RNA was prepared in a similar fashion. A BHK cell suspension in PBS (0.8 mL, 1.2 x 10⁷ cells) was mixed with 10 µg of each RNA, and the mixture was electroporated. Electroporation settings for Gene-Pulser® (Bio-Rad Laboratories) were: 850 V, 25 µF, 3 pulses. Culture supernatant was collected at 18-24 hr post-electroporation and clarified by centrifugation for 10 min at 1000 rpm.

15

Titration of Virosomes

The presence of infectious virosome particles was demonstrated using an immunofluorescence assay to titer the virosomes by detecting the fluorescence of the GFP encoded by the replicon RNA in the virosomes. Serial dilutions of the cell culture supernatant were added to 12-well plates of BHK cells. Following an 18-24 hour incubation in an atmosphere of 5% CO₂ at 37°C, the medium was removed from each plate. Virosome infectious titer was then determined by counting the number of green-fluorescent single cells at a particular dilution, followed by a back-calculation to determine total infectious units (i.u.) per mL. A final titer of 440 i.u./mL was collected.

Confirmation of virosome identity

Three independent experimental methods were used to determine that the infectious particles were in fact virosomes, rather than replication competent viral

particles or naked RNA being carried over from the electroporated cells.

5 i) The virosome-containing supernatant was passaged a second time by removing the cell supernatant from the 12-well plate used for titration and placing this supernatant onto a fresh monolayer of BHK cells. At 18-24 hours post-passage, the monolayer was examined under U/V fluorescence and found to contain 0 (zero) GFP-positive cells, indicating the infectious particles produced using this method can undergo only a single round of replication, a critical characteristic of a virosome.

10 ii) To establish that the infectious titer detected following virosome packaging was not due to carry-over of RNA used in the electroporation, the supernatant was treated with RNase A (Invitrogen) at a concentration of 100 μ g/ μ L for 15 minutes at 37°C. The treated and untreated control supernatants were titered according to the methods outlined above. The RNase-treated sample contained 400 i.u./mL and the control group had 440 i.u./mL, indicating that the RNase treatment had no significant effect on virosome titer.

15 iii) To establish that the infectious particles were enveloped in the E1 and E2 glycoproteins, anti-VEE mouse serum was used to treat the cell supernatant in a neutralization assay. As a control, normal mouse serum was used to treat the virosome supernatant. In addition, VEE replicon particles expressing GFP were used in the assay, the infectivity of which 20 is known to be inhibited by this serum.

25	Anti-VEE serum	Particle Titer (i.u./mL)	
		Normal Mouse	No sen
Virosome Supernatant	20	440	530
VRP-GFP	0	530	890

30 The infectivity of the virosomes was inhibited similar to that of VRP-GFP,

indicating that the virosome particles were enveloped by the E1 and E2 glycoproteins.

These examples clearly demonstrate the ability to produce infectious virosome particles comprising replicon RNA enveloped with only the alphavirus E1 and E2 glycoproteins. Testing confirmed that these virosomes are infectious agents, but that they undergo only a single round of replication, as indicated by the inability to passage the agent. In addition, the agents contained the E1 and E2 glycoproteins, as evidenced by the ability to block infection with only VEE specific serum. Finally, the infectious RNA is protected from RNase enzymatic digestion, indicating an enveloped particle.

10

The natural lipid content in BHK cells is primarily non-cationic. Virosomes made in a completely cell free system can be made by using one or more non-cationic lipids, such as lecithin (phosphatidylcholine).

15

EXAMPLE 10

PHASE I CLINICAL PROTOCOL

Phase I Safety and Immunogenicity Trial of an HIV Subtype C Gag-VEE

20 **Replicon Particle Vaccine in HIV-1 Seronegative Human Subjects**

A Phase I trial is conducted to evaluate the safety and immunogenicity of the HIV Gag-VRP prototype vaccine component in healthy seronegative adult volunteers. The doses are selected based on preclinical studies in rodents and nonhuman primates. 25 The schedule mimics previous preclinical efficacy studies with the SIV model that demonstrated the capacity of SIV-VRP to induce SIV specific neutralizing antibodies and CTL.

Purpose: To evaluate the candidate vaccine component in an open-labeled, 30 placebo-controlled study.

Subjects: Healthy adult volunteers without a history of identifiable high-risk behavior for HIV-1 infection as determined by a comprehensive screening questionnaire.

No. Subjects: 40

5

Route: Subcutaneous injection

Scheme: The volunteers are arranged in four groups, ten subjects per group. In each group, two subjects receive a placebo, while the other eight subjects receive either 10^4 , 10^6 , 10^7 , or 10^8 i.u. of HIV-Gag-VRPs. Subjects are vaccinated on day 0, day 30, and 10 day 120.

Estimated Duration: Forty weeks

A. SELECTION of SUBJECTS

15 Subjects are healthy HIV-1 seronegative adults who fully comprehend the purpose and details of the study as described in the informed consent. Subjects whom either themselves or whose sexual partners have identifiable higher risk behavior for HIV-1 infection are not eligible. Higher risk behavior is determined by a prescreen series of questions designed to identify risk factors for HIV-1 infection. An assessment 20 of absolute exclusion criteria using the self-administered and interview questions is conducted. Subsequently, investigators proceed with phlebotomy, history and physical examination, and final questions regarding sexual behavior and other practices. Eligibility determinations for the trial depend on results of laboratory tests and answers to these self-administered and interview questions.

25

The criteria used to define low risk behavior are as follows:

EITHER ALL OF THE FOLLOWING:

1. No newly acquired higher risk associated STD in the last six months
- 30 2. No possibly safe or unsafe sex with a known HIV+ individual or an active

injection drug user in the past six months

3. No unsafe sexual activity
4. Possibly safe sexual activity with two or fewer partners within the last six months
5. No injection drug use

OR BOTH OF THE FOLLOWING:

1. Mutually monogamous relationship with a known or presumed HIV seronegative partner for the last six months
- 10 2. No injection drug use

A.1 Inclusion Criteria

Age: 18-60

- 15 Sex: Male or Female *[For females, negative pregnancy test at time of entry and assurance that adequate birth control measures will be used for one month prior to immunization and the duration of the study]*
Normal history and physical examination
Lower risk sexual behavior as defined above.
- 20 Normal complete blood count and differential defined as:
 - Hematocrit 34% for women; 38% for men
 - White count 3500 cells/mm³ with normal differential
 - Total lymphocyte count 800 cells/mm³
 - Absolute CD4 count 400 cells/mm³
- 25 - Platelets (150,000-550,000)
Normal ALT (~ 1.5 x institutional upper normal limit) and creatinine (1.6 mg/dl)
Normal urine dipstick with esterase and nitrite
Negative for hepatitis B surface antigen
- 30 Negative ELISA for HIV within eight weeks of immunization

Availability for follow-up for planned duration of the study (68 weeks)

A viable EBV transformed autologous B cell line

A.2 Exclusion Criteria

5

History of immunodeficiency, chronic illness, malignancy, autoimmune disease, or use of immunosuppressive medications

10 Medical or psychiatric condition or occupational responsibilities which preclude subject compliance with the protocol

Subjects with identifiable higher risk behavior for HIV infection as determined by screening questionnaire designed to identify risk factors for HIV infection; specific exclusions include:

15 History of injection drug use within the last 12 months prior to enrollment.

Higher risk sexual behavior defined as one or more of the following behaviors:

1. A newly acquired higher risk associated STD within the past six months
2. Possibly safe or unsafe sex with a known HIV+ individual in the past six months

20 3. Possibly safe sexual activity with twelve or more partners in the past six months

4. Unsafe sexual activity with four or more partners within the past six months.

25 Live attenuated vaccines within 60 days of study [NOTE: Medically indicated subunit or killed vaccines (e.g., influenza, pneumococcal) are not exclusionary, but should be given at least two weeks away from test article immunizations.]

Use of experimental agents within 30 days prior to study

Receipt of blood products or immunoglobulin in the past six months

30 Active syphilis [NOTE: If the serology is documented to be a false positive or

due to a remote (>six months) treated infection, the volunteer is eligible]

Active tuberculosis [NOTE: Volunteers with a positive PPD and a normal chest X-ray showing no evidence of TB and not requiring INH therapy are eligible.]

History of anaphylaxis or other serious adverse reactions to vaccines

5 Prior receipt of HIV vaccines or a placebo recipient in an HIV vaccine trial

Pregnant or lactating women

B. SAFETY and IMMUNOGENICITY MONITORING

10 Safety is evaluated by monitoring volunteers for adverse reactions during the course of the trial. Volunteers are followed for a total of 26 weeks post-final inoculation. The main toxicity associated with the subcutaneous injection in this study is that associated with subcutaneous injection of any immunogen, i.e., pain, redness and swelling at the injection site, as well as the possibility of fever, chills, aches and pains
15 and perhaps fatigue.

Safety monitoring includes periodic review of data from the trial with particular emphasis on monitoring for adverse reactions including the following evaluations:

Hematologic: CBC, differential, platelets

20 Hepatic/renal: ALT, creatinine, urinalysis

Neurologic: headache, paralysis, anxiety, confusion, weakness, tremors.

Systemic symptoms: fever, gastrointestinal complaints, myalgia, malaise, fatigue, headache, anaphylaxis, immune complex disease, and other hypersensitivity reactions

25 Local toxicity at the site of injection: e.g., pain, tenderness, erythema, regional lymphadenopathy, limitation of limb movement

The immunogenicity monitoring includes the following immunological assays, all utilizing HIV Subtype C based reagents:

Humoral responses:

HIV Subtype C Gag-specific ELISA
Anti-VEE ELISA

5 Cellular immune responses:

Standard cell-killing assay (i.e., chromium release) to measure CD8+ Gag-specific CTL activity

ELISPOT assay to measure IFN-?

10 Mucosal immune responses:

Standardized assay for assessment of Gag-specific IgA

Throughout this application, various publications are referenced. The disclosures of these publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art to which this invention pertains.

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What is claimed is:

1. A composition comprising two or more isolated nucleic acids selected from the group consisting of an isolated nucleic acid encoding an *env* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, an isolated nucleic acid encoding a *gag* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, wherein the *gag* gene product or immunogenic fragment thereof is modified to inhibit formation of virus-like particles containing the *gag* gene product or the immunogenic fragment thereof and their release from a cell, and an isolated nucleic acid encoding a *pol* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, wherein the *pol* gene product or immunogenic fragment thereof is modified to inhibit reverse transcriptase activity.
2. A composition comprising a population of alphavirus replicon particles comprising two or more isolated nucleic acids selected from the group consisting of 1) an isolated nucleic acid encoding an *env* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, 2) an isolated nucleic acid encoding a *gag* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, wherein the *gag* gene product or immunogenic fragment thereof is modified to inhibit formation of virus-like particles containing the *gag* gene product or the immunogenic fragment thereof and their release from a cell, and 3) an isolated nucleic acid encoding a *pol* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, wherein the *pol* gene product or immunogenic fragment thereof is modified to inhibit reverse transcriptase activity, and wherein the nucleic acids are each contained within a separate alphavirus replicon particle.
3. A composition comprising a population of alphavirus replicon particles comprising two or more isolated nucleic acids selected from the group consisting of 1) an isolated nucleic acid encoding an *env* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, 2) an isolated nucleic acid encoding a *gag*

gene product or an immunogenic fragment thereof of a human immunodeficiency virus, wherein the *gag* gene product or immunogenic fragment thereof is modified to inhibit formation of virus-like particles containing the *gag* gene product or the immunogenic fragment thereof and their release from a cell, and 3) an isolated nucleic acid encoding a *pol* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, wherein the *pol* gene product or immunogenic fragment thereof is modified to inhibit reverse transcriptase activity, and wherein the nucleic acids are each contained within a separate alphavirus replicon particle, and further wherein the alphavirus replicon particles comprise a replicon RNA or at least one structural protein which comprises one or more attenuating mutations.

4. A method of making the population of alphavirus replicon particles of claim 2 comprising:
 - A) (a) providing a first helper cell for producing a first population of infectious, replication defective alphavirus particles, comprising in an alphavirus-permissive cell:
 - (i) an alphavirus replicon RNA, wherein the replicon RNA comprises an alphavirus packaging signal and a nucleic acid encoding an *env* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, and wherein the replicon RNA lacks sequences encoding alphavirus structural proteins;
 - (ii) a first helper RNA separate from said replicon RNA, said first helper RNA encoding at least one alphavirus structural protein and furthermore not encoding at least one other alphavirus structural protein; and
 - (iii) one or more additional helper RNA(s) separate from said replicon RNA and separate from said first helper RNA, said additional helper RNA(s) encoding at least one other alphavirus structural protein not encoded by said first helper RNA;

and with at least one of said helper RNAs lacking an alphavirus packaging signal;

wherein the combined expression of the alphavirus replicon RNA and the helper RNAs produces an assembled alphavirus particle which is able to infect a cell, and is unable to complete viral replication, and further wherein the first population contains no detectable replication-competent alphavirus particles as determined by passage on permissive cells in culture;

- (b) producing the alphavirus particles in the helper cell; and
- (c) collecting the alphavirus particles from the helper cells;

B) (a) providing a second helper cell for producing a second population of infectious, replication defective alphavirus particles, comprising in an alphavirus-permissive cell:

- (i) an alphavirus replicon RNA, wherein the replicon RNA comprises an alphavirus packaging signal and a nucleic acid encoding a *gag* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, wherein the *gag* gene product or immunogenic fragment thereof is modified to inhibit formation of virus-like particles containing the *gag* gene product or the immunogenic fragment thereof and their release from a cell, and wherein the replicon RNA lacks sequences encoding alphavirus structural proteins;

- (ii) a first helper RNA separate from said replicon RNA, said first helper RNA encoding at least one alphavirus structural protein and furthermore not encoding at least one other alphavirus structural protein; and

- (iii) one or more additional helper RNA(s) separate from said replicon RNA and separate from said first helper RNA, said additional helper RNA(s) encoding at least one other alphavirus structural protein not encoded by said first helper RNA;

and with at least one of said helper RNAs lacking an alphavirus packaging signal;

wherein the combined expression of the alphavirus replicon RNA and the

helper RNAs produces an assembled alphavirus particle which is able to infect a cell, and is unable to complete viral replication, and further wherein the second population contains no detectable replication-competent alphavirus particles as determined by passage on permissive cells in culture;

- (b) producing the alphavirus particles in the helper cell; and
- (c) collecting the alphavirus particles from the helper cells;

C) (a) providing a third helper cell for producing a third population of infectious, replication defective alphavirus particles, comprising in an alphavirus-permissive cell:

- (i) an alphavirus replicon RNA, wherein the replicon RNA comprises an alphavirus packaging signal and a nucleic acid encoding a *pol* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, wherein the *pol* gene product or immunogenic fragment thereof is modified to inhibit reverse transcriptase activity, and wherein the replicon RNA lacks sequences encoding alphavirus structural proteins;

- (ii) a first helper RNA separate from said replicon RNA, said first helper RNA encoding at least one alphavirus structural protein and furthermore not encoding at least one other alphavirus structural protein; and

- (iii) one or more additional helper RNA(s) separate from said replicon RNA and separate from said first helper RNA, said additional helper RNA(s) encoding at least one other alphavirus structural protein not encoded by said first helper RNA;

and with at least one of said helper RNAs lacking an alphavirus packaging signal;

wherein the combined expression of the alphavirus replicon RNA and the helper RNAs produces an assembled alphavirus particle which is able to infect a cell, and is unable to complete viral replication, and further wherein the third population contains

no detectable replication-competent alphavirus particles as determined by passage on permissive cells in culture;

- (b) producing the alphavirus particles in the helper cell; and
- (c) collecting the alphavirus particles from the helper cells; and

D) combining the first population of alphavirus particles produced from the first helper cell, the second population of alphavirus particles produced from the second helper cell and the third population of alphavirus particles produced from the third helper cell, thereby producing the population of alphavirus replicon particles of claim 2.

5. The method of claim 4, wherein the alphavirus replicon RNA of at least one of the first helper cell, the second helper cell and the third helper cell comprises sequence encoding at least one alphavirus structural protein and wherein the first helper RNA and the one or more additional helper RNA(s) in the at least one of the first helper cell, the second helper cell and the third helper cell, encodes at least one other alphavirus structural protein not encoded by said replicon RNA.

6. A method of making the population of alphavirus replicon particles of claim 3, comprising:

A) (a) providing a first helper cell for producing a first population of infectious, replication defective alphavirus particles, comprising in an alphavirus-permissive cell:

- (i) an alphavirus replicon RNA, wherein the replicon RNA comprises an alphavirus packaging signal and a nucleic acid encoding an *env* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, and wherein the replicon RNA lacks sequences encoding alphavirus structural proteins;

- (ii) a first helper RNA separate from said replicon RNA, said first helper RNA encoding at least one alphavirus structural protein and furthermore not encoding at least one other alphavirus structural protein;

and

(iii) one or more additional helper RNA(s) separate from said replicon RNA and separate from said first helper RNA, said additional helper RNA(s) encoding at least one other alphavirus structural protein not encoded by said first helper RNA;

and with at least one of said helper RNAs lacking an alphavirus packaging signal;

wherein the combined expression of the alphavirus replicon RNA and the helper RNAs produces an assembled alphavirus particle which is able to infect a cell, and is unable to complete viral replication, and further wherein the first population contains no detectable replication-competent alphavirus particles as determined by passage on permissive cells in culture, and further wherein at least one of said replicon RNA, said first helper RNA, and said one or more additional helper RNA(s) comprises one or more attenuating mutations;

- (b) producing the alphavirus particles in the helper cell; and
- (c) collecting the alphavirus particles from the helper cells;

B) (a) providing a second helper cell for producing a second population of infectious, replication defective alphavirus particle, comprising in an alphavirus-permissive cell:

- (i) an alphavirus replicon RNA, wherein the replicon RNA comprises an alphavirus packaging signal and a nucleic acid encoding a *gag* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, wherein the *gag* gene product or immunogenic fragment thereof is modified to inhibit formation of virus-like particles containing the *gag* gene product or the immunogenic fragment thereof and their release from a cell, and wherein the replicon RNA lacks sequences encoding alphavirus structural proteins;
- (ii) a first helper RNA separate from said replicon RNA, said first helper RNA encoding at least one alphavirus structural protein and

furthermore not encoding at least one other alphavirus structural protein; and

(iii) one or more additional helper RNA(s) separate from said replicon RNA and separate from said first helper RNA, said additional helper RNA(s) encoding at least one other alphavirus structural protein not encoded by said first helper RNA;

and with at least one of said helper RNAs lacking an alphavirus packaging signal;

wherein the combined expression of the alphavirus replicon RNA and the helper RNAs produces an assembled alphavirus particle which is able to infect a cell, and is unable to complete viral replication, and further wherein the second population contains no detectable replication-competent alphavirus particles as determined by passage on permissive cells in culture, and further wherein at least one of said replicon RNA, said first helper RNA, and said one or more additional helper RNA(s) comprises one or more attenuating mutations;

- (b) producing the alphavirus particles in the helper cell; and
- (c) collecting the alphavirus particles from the helper cells;

C) (a) providing a third helper cell for producing a third population of infectious, replication defective alphavirus particles, comprising in an alphavirus-permissive cell:

(i) an alphavirus replicon RNA, wherein the replicon RNA comprises an alphavirus packaging signal and a nucleic acid encoding a *pol* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, wherein the *pol* gene product or immunogenic fragment thereof is modified to inhibit reverse transcriptase activity, and wherein the replicon RNA lacks sequences encoding alphavirus structural proteins;

(ii) a first helper RNA separate from said replicon RNA, said first helper RNA encoding at least one alphavirus structural protein and

furthermore not encoding at least one other alphavirus structural protein; and

(iii) one or more additional helper RNA(s) separate from said replicon RNA and separate from said first helper RNA, said additional helper RNA(s) encoding at least one other alphavirus structural protein not encoded by said first helper RNA;

and with at least one of said helper RNAs lacking an alphavirus packaging signal;

wherein the combined expression of the alphavirus replicon RNA and the helper RNAs produces an assembled alphavirus particle which is able to infect a cell, and is unable to complete viral replication, and further wherein the third population contains no detectable replication-competent alphavirus particles as determined by passage on permissive cells in culture, and further wherein at least one of said replicon RNA, said first helper RNA, and said one or more additional helper RNA(s) comprises one or more attenuating mutations;

- (b) producing the alphavirus particles in the helper cell; and
- (c) collecting the alphavirus particles from the helper cells; and

D) combining the first population of alphavirus particles produced from the first helper cell, the second population of alphavirus particles produced from the second helper cell and the third population of alphavirus particles produced from the third helper cell, thereby producing the population of alphavirus replicon particles of claim 3.

7. The method of claim 6, wherein the alphavirus replicon RNA of at least one of the first helper cell, the second helper cell and the third helper cell comprises sequence encoding at least one alphavirus structural protein and wherein the first helper RNA and the one or more additional helper RNA(s) in the at least one of the first helper cell, the second helper cell and the third helper cell, encodes at least one other alphavirus structural protein not encoded by said replicon RNA.

8. The method of claim 6, wherein only at least one of the first population of alphavirus particles, the second population of alphavirus particles and the third population of alphavirus particles comprises particles wherein at least one of said replicon RNA, said first helper RNA, and said one or more additional helper RNA(s) comprises one or more attenuating mutations.
9. A population of alphavirus replicon particles produced by the method of claim 4.
10. A population of alphavirus replicon particles produced by the method of claim 6.
11. A method of inducing an immune response to human immunodeficiency virus in a subject, comprising administering to the subject an immunogenic amount of the composition of claim 1 in a pharmaceutically acceptable carrier.
12. A method of inducing an immune response to human immunodeficiency virus in a subject, comprising administering to the subject an immunogenic amount of the composition of claim 2 in a pharmaceutically acceptable carrier.
13. A method of inducing an immune response to human immunodeficiency virus in a subject, comprising administering to the subject an immunogenic amount of the composition of claim 3 in a pharmaceutically acceptable carrier.
14. A method of inducing an immune response to human immunodeficiency virus in a subject, comprising administering to the subject an immunogenic amount of the population of claim 9 in a pharmaceutically acceptable carrier.
15. A method of inducing an immune response to human immunodeficiency virus in a subject, comprising administering to the subject an immunogenic amount of the

population of claim 10 in a pharmaceutically acceptable carrier.

16. A method of treating or preventing infection by human immunodeficiency virus in a subject, comprising administering to the subject an immunogenic amount of the composition of claim 1 in a pharmaceutically acceptable carrier.

17. A method of treating or preventing infection by human immunodeficiency virus in a subject, comprising administering to the subject an immunogenic amount of the composition of claim 2 in a pharmaceutically acceptable carrier.

18. A method of treating or preventing infection by human immunodeficiency virus in a subject, comprising administering to the subject an immunogenic amount of the composition of claim 3 in a pharmaceutically acceptable carrier.

19. A method of treating or preventing infection by human immunodeficiency virus in a subject, comprising administering to the subject an immunogenic amount of the population of claim 9 in a pharmaceutically acceptable carrier.

20. A method of treating or preventing infection by human immunodeficiency virus in a subject, comprising administering to the subject an immunogenic amount of the population of claim 10 in a pharmaceutically acceptable carrier.

21. A composition comprising two or more isolated nucleic acids selected from the group consisting of an isolated nucleic acid encoding an *env* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, an isolated nucleic acid encoding a *gag* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, wherein the *gag* gene product or immunogenic fragment thereof is modified to inhibit formation of virus-like particles containing the *gag* gene product or the immunogenic fragment thereof and their release from a cell, and an isolated nucleic acid encoding a *pol* gene product or an immunogenic fragment thereof

of a human immunodeficiency virus, wherein the *pol* gene product or immunogenic fragment thereof comprises a modification resulting in deletion or inactivation of integrase, RNase H and reverse transcriptase functions in the *pol* gene product or immunogenic fragment thereof.

22. A composition comprising a population of alphavirus replicon particles comprising two or more isolated nucleic acids selected from the group consisting of 1) an isolated nucleic acid encoding an *env* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, 2) an isolated nucleic acid encoding a *gag* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, wherein the *gag* gene product or immunogenic fragment thereof is modified to inhibit formation of virus-like particles containing the *gag* gene product or the immunogenic fragment thereof and their release from a cell, and 3) an isolated nucleic acid encoding a *pol* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, wherein the *pol* gene product or immunogenic fragment thereof comprises a modification resulting in deletion or inactivation of integrase, RNase H and reverse transcriptase functions in the *pol* gene product or immunogenic fragment thereof, and wherein the nucleic acids are each contained within a separate alphavirus replicon particle.

23. A composition comprising a population of alphavirus replicon particles comprising two or more isolated nucleic acids selected from the group consisting of 1) an isolated nucleic acid encoding an *env* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, 2) an isolated nucleic acid encoding a *gag* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, wherein the *gag* gene product or immunogenic fragment thereof is modified to inhibit formation of virus-like particles containing the *gag* gene product or the immunogenic fragment thereof and their release from a cell, and 3) an isolated nucleic acid encoding a *pol* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, wherein the *pol* gene product or immunogenic fragment thereof comprises a

modification resulting in deletion or inactivation of integrase, RNase H and reverse transcriptase functions in the *pol* gene product or immunogenic fragment thereof, and wherein the nucleic acids are each contained within a separate alphavirus replicon particle, and further wherein the alphavirus replicon particles comprise a replicon RNA or at least one structural protein which comprises one or more attenuating mutations.

24. A method of making the population of alphavirus replicon particles of claim 22, comprising:

A) (a) providing a first helper cell for producing a first population of infectious, replication defective alphavirus particles, comprising in an alphavirus-permissive cell:

(i) an alphavirus replicon RNA, wherein the replicon RNA comprises an alphavirus packaging signal and a nucleic acid encoding an *env* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, and wherein the replicon RNA lacks sequences encoding alphavirus structural proteins;

(ii) a first helper RNA separate from said replicon RNA, said first helper RNA encoding at least one alphavirus structural protein and furthermore not encoding at least one other alphavirus structural protein; and

(iii) one or more additional helper RNA(s) separate from said replicon RNA and separate from said first helper RNA, said additional helper RNA(s) encoding at least one other alphavirus structural protein not encoded by said first helper RNA;

and with at least one of said helper RNAs lacking an alphavirus packaging signal;

wherein the combined expression of the alphavirus replicon RNA and the helper RNAs produces an assembled alphavirus particle which is able to infect a cell, and is unable to complete viral replication, and further wherein the first population contains no detectable replication-competent alphavirus particles as determined by passage on permissive cells in culture;

- (b) producing the alphavirus particles in the helper cell; and
- (c) collecting the alphavirus particles from the helper cells;

B) (a) providing a second helper cell for producing a second population of infectious, replication defective alphavirus particles, comprising in an alphavirus-permissive cell:

- (i) an alphavirus replicon RNA, wherein the replicon RNA comprises an alphavirus packaging signal and a nucleic acid encoding a *gag* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, wherein the *gag* gene product or immunogenic fragment thereof is modified to inhibit formation of virus-like particles containing the *gag* gene product or the immunogenic fragment thereof and their release from a cell, and wherein the replicon RNA lacks sequences encoding alphavirus structural proteins;
- (ii) a first helper RNA separate from said replicon RNA, said first helper RNA encoding at least one alphavirus structural protein and furthermore not encoding at least one other alphavirus structural protein; and
- (iii) one or more additional helper RNA(s) separate from said replicon RNA and separate from said first helper RNA, said additional helper RNA(s) encoding at least one other alphavirus structural protein not encoded by said first helper RNA;

and with at least one of said helper RNAs lacking an alphavirus packaging signal;

wherein the combined expression of the alphavirus replicon RNA and the helper RNAs produces an assembled alphavirus particle which is able to infect a cell, and is unable to complete viral replication, and further wherein the second population contains no detectable replication-competent alphavirus particles as determined by passage on permissive cells in culture;

- (b) producing the alphavirus particles in the helper cell; and

(c) collecting the alphavirus particles from the helper cells;

C) (a) providing a third helper cell for producing a third population of infectious, replication defective alphavirus particles, comprising in an alphavirus-permissive cell:

(i) an alphavirus replicon RNA, wherein the replicon RNA comprises an alphavirus packaging signal and a nucleic acid encoding a *pol* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, wherein the *pol* gene product or immunogenic fragment thereof comprises a modification resulting in deletion or inactivation of integrase, RNase H and reverse transcriptase functions in the *pol* gene product or immunogenic fragment thereof, and wherein the replicon RNA lacks sequences encoding alphavirus structural proteins;

(ii) a first helper RNA separate from said replicon RNA, said first helper RNA encoding at least one alphavirus structural protein and furthermore not encoding at least one other alphavirus structural protein; and

(iii) one or more additional helper RNA(s) separate from said replicon RNA and separate from said first helper RNA, said additional helper RNA(s) encoding at least one other alphavirus structural protein not encoded by said first helper RNA;

and with at least one of said helper RNAs lacking an alphavirus packaging signal;

wherein the combined expression of the alphavirus replicon RNA and the helper RNAs produces an assembled alphavirus particle which is able to infect a cell, and is unable to complete viral replication, and further wherein the third population contains no detectable replication-competent alphavirus particles as determined by passage on permissive cells in culture;

(b) producing the alphavirus particles in the helper cell; and

(c) collecting the alphavirus particles from the helper cells; and

D) combining the first population of alphavirus particles produced from the first helper cell, the second population of alphavirus particles produced from the second helper cell and the third population of alphavirus particles produced from the third helper cell, thereby producing the population of alphavirus replicon particles of claim 22.

25. The method of claim 24, wherein the alphavirus replicon RNA of at least one of the first helper cell, the second helper cell and the third helper cell comprises sequence encoding at least one alphavirus structural protein and wherein the first helper RNA and the one or more additional helper RNA(s) in the at least one of the first helper cell, the second helper cell and the third helper cell, encodes at least one other alphavirus structural protein not encoded by said replicon RNA.

26. A method of making the population of alphavirus replicon particles of claim 23, comprising:

A) (a) providing a first helper cell for producing a first population of infectious, replication defective alphavirus particles, comprising in an alphavirus-permissive cell:

- (i) an alphavirus replicon RNA, wherein the replicon RNA comprises an alphavirus packaging signal and a nucleic acid encoding an *env* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, and wherein the replicon RNA lacks sequences encoding alphavirus structural proteins;
- (ii) a first helper RNA separate from said replicon RNA, said first helper RNA encoding at least one alphavirus structural protein and furthermore not encoding at least one other alphavirus structural protein; and
- (iii) one or more additional helper RNA(s) separate from said replicon RNA and separate from said first helper RNA, said additional helper RNA(s) encoding at least one other alphavirus structural protein not encoded by said first helper RNA;

and with at least one of said helper RNAs lacking an alphavirus packaging signal;

wherein the combined expression of the alphavirus replicon RNA and the helper RNAs produces an assembled alphavirus particle which is able to infect a cell, and is unable to complete viral replication, and further wherein the first population contains no detectable replication-competent alphavirus particles as determined by passage on permissive cells in culture, and further wherein at least one of said replicon RNA, said first helper RNA, and said one or more additional helper RNA(s) comprises one or more attenuating mutations;

- (b) producing the alphavirus particles in the helper cell; and
- (c) collecting the alphavirus particles from the helper cells;

B) (a) providing a second helper cell for producing a second population of infectious, replication defective alphavirus particle, comprising in an alphavirus-permissive cell:

- (i) an alphavirus replicon RNA, wherein the replicon RNA comprises an alphavirus packaging signal and a nucleic acid encoding a *gag* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, wherein the *gag* gene product or immunogenic fragment thereof is modified to inhibit formation of virus-like particles containing the *gag* gene product or the immunogenic fragment thereof and their release from a cell, and wherein the replicon RNA lacks sequences encoding alphavirus structural proteins;
- (ii) a first helper RNA separate from said replicon RNA, said first helper RNA encoding at least one alphavirus structural protein and furthermore not-encoding at least one other alphavirus structural protein; and
- (iii) one or more additional helper RNA(s) separate from said replicon RNA and separate from said first helper RNA, said additional helper RNA(s) encoding at least one other alphavirus structural protein

not encoded by said first helper RNA;
and with at least one of said helper RNAs lacking an alphavirus packaging signal;

wherein the combined expression of the alphavirus replicon RNA and the helper RNAs produces an assembled alphavirus particle which is able to infect a cell, and is unable to complete viral replication, and further wherein the second population contains no detectable replication-competent alphavirus particles as determined by passage on permissive cells in culture, and further wherein at least one of said replicon RNA, said first helper RNA, and said one or more additional helper RNA(s) comprises one or more attenuating mutations;

- (b) producing the alphavirus particles in the helper cell; and
- (c) collecting the alphavirus particles from the helper cells;

C) (a) providing a third helper cell for producing a third population of infectious, replication defective alphavirus particles, comprising in an alphavirus-permissive cell:

- (i) an alphavirus replicon RNA, wherein the replicon RNA comprises an alphavirus packaging signal and a nucleic acid encoding a *pol* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, wherein the *pol* gene product or immunogenic fragment thereof comprises a modification resulting in deletion or inactivation of integrase, RNase H and reverse transcriptase functions in the *pol* gene product or immunogenic fragment thereof, and wherein the replicon RNA lacks sequences encoding alphavirus structural proteins;
- (ii) a first helper RNA separate from said replicon RNA, said first helper RNA encoding at least one alphavirus structural protein and furthermore not encoding at least one other alphavirus structural protein; and
- (iii) one or more additional helper RNA(s) separate from said replicon RNA and separate from said first helper RNA, said additional

helper RNA(s) encoding at least one other alphavirus structural protein not encoded by said first helper RNA; and with at least one of said helper RNAs lacking an alphavirus packaging signal;

wherein the combined expression of the alphavirus replicon RNA and the helper RNAs produces an assembled alphavirus particle which is able to infect a cell, and is unable to complete viral replication, and further wherein the third population contains no detectable replication-competent alphavirus particles as determined by passage on permissive cells in culture, and further wherein at least one of said replicon RNA, said first helper RNA, and said one or more additional helper RNA(s) comprises one or more attenuating mutations;

- (b) producing the alphavirus particles in the helper cell; and
- (c) collecting the alphavirus particles from the helper cells; and

D) combining the first population of alphavirus particles produced from the first helper cell, the second population of alphavirus particles produced from the second helper cell and the third population of alphavirus particles produced from the third helper cell, thereby producing the population of alphavirus replicon particles of claim 23.

27. The method of claim 26, wherein the alphavirus replicon RNA of at least one of the first helper cell, the second helper cell and the third helper cell comprises sequence encoding at least one alphavirus structural protein and wherein the first helper RNA and the one or more additional helper RNA(s) in the at least one of the first helper cell, the second helper cell and the third helper cell, encodes at least one other alphavirus structural protein not encoded by said replicon RNA.

28. The method of claim 26, wherein only at least one of the first population of alphavirus particles, the second population of alphavirus particles and the third population of alphavirus particles comprises particles wherein at least one of said

, said first helper RNA, and said one or more additional helper RNA(s) or more attenuating mutations.

ulation of alphavirus replicon particles produced by the method of claim

ulation of alphavirus replicon particles produced by the method of claim

ethod of inducing an immune response to human immunodeficiency virus comprising administering to the subject an immunogenic amount of the of claim 21 in a pharmaceutically acceptable carrier.

ethod of inducing an immune response to human immunodeficiency virus comprising administering to the subject an immunogenic amount of the of claim 22 in a pharmaceutically acceptable carrier.

ethod of inducing an immune response to human immunodeficiency virus comprising administering to the subject an immunogenic amount of the of claim 23 in a pharmaceutically acceptable carrier.

ethod of inducing an immune response to human immunodeficiency virus comprising administering to the subject an immunogenic amount of the of claim 29 in a pharmaceutically acceptable carrier.

ethod of inducing an immune response to human immunodeficiency virus comprising administering to the subject an immunogenic amount of the of claim 30 in a pharmaceutically acceptable carrier.

ethod of treating or preventing infection by human immunodeficiency

virus in a subject, comprising administering to the subject an immunogenic amount of the composition of claim 21 in a pharmaceutically acceptable carrier.

37. A method of treating or preventing infection by human immunodeficiency virus in a subject, comprising administering to the subject an immunogenic amount of the composition of claim 22 in a pharmaceutically acceptable carrier.

38. A method of treating or preventing infection by human immunodeficiency virus in a subject, comprising administering to the subject an immunogenic amount of the composition of claim 23 in a pharmaceutically acceptable carrier.

39. A method of treating or preventing infection by human immunodeficiency virus in a subject, comprising administering to the subject an immunogenic amount of the population of claim 29 in a pharmaceutically acceptable carrier.

40. A method of treating or preventing infection by human immunodeficiency virus in a subject, comprising administering to the subject an immunogenic amount of the population of claim 30 in a pharmaceutically acceptable carrier.

41. An alphavirus replicon viosome comprising an alphavirus replicon RNA encapsidated by a lipid bilayer comprising alphavirus glycoproteins, E1 and E2.

42. The viosome of claim 41, wherein the alphavirus glycoproteins are Venezuelan Equine Encephalitis glycoproteins E1 and E2.

43. A method of producing the alphavirus replicon viosome of claim 41, comprising:

a) combining alphavirus replicon RNA, alphavirus glycoproteins E1 and E2, non-cationic lipids and detergent; and

b) gradually removing detergent, whereby alphavirus replicon virosomes are produced.

44. An alphavirus replicon virosome produced from the method of claim 43.

45. A method of eliciting an immune response in a subject, comprising administering to the subject an immunogenic amount of the alphavirus replicon virosome of claim 41 in a pharmaceutically acceptable carrier.

46. A method of treating or preventing infection by human immunodeficiency virus in a subject, comprising administering to the subject an immunogenic amount of the alphavirus replicon virosome of claim 41, wherein the virosome comprises alphavirus replicon RNA encoding one or more HIV immunogens.

47. A composition comprising a population of alphavirus replicon virosomes comprising two or more isolated nucleic acids selected from the group consisting of 1) an isolated nucleic acid encoding an *env* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, 2) an isolated nucleic acid encoding a *gag* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, wherein the *gag* gene product or immunogenic fragment thereof is modified to inhibit formation of virus-like particles containing the *gag* gene product or the immunogenic fragment thereof and their release from a cell, and 3) an isolated nucleic acid encoding a *pol* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, wherein the *pol* gene product or immunogenic fragment thereof comprises a modification resulting in deletion or inactivation of integrase, RNase H and reverse transcriptase functions in the *pol* gene product or immunogenic fragment thereof, and wherein the nucleic acids are each contained within a separate alphavirus replicon virosome.

48. A composition comprising a population of alphavirus replicon virosomes

comprising two or more isolated nucleic acids selected from the group consisting of 1) an isolated nucleic acid encoding an *env* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, 2) an isolated nucleic acid encoding a *gag* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, wherein the *gag* gene product or immunogenic fragment thereof is modified to inhibit formation of virus-like particles containing the *gag* gene product or the immunogenic fragment thereof and their release from a cell, and 3) an isolated nucleic acid encoding a *pol* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, wherein the *pol* gene product or immunogenic fragment thereof comprises a modification resulting in inactivation of reverse transcriptase activity in the *pol* gene product or immunogenic fragment thereof, and wherein the nucleic acids are each contained within a separate alphavirus replicon virosome.

49. A method of producing the population of alphavirus replicon virosomes of claim 47, comprising:

A) (a) producing a first population of alphavirus replicon virosomes by combining alphavirus replicon RNA comprising nucleic acid encoding and *env* gene product or immunogenic fragment thereof, alphavirus glycoproteins E1 and E2, non-cationic lipids and detergent; and

b) gradually removing detergent, whereby alphavirus replicon virosomes are produced;

B) (a) producing a second population of alphavirus replicon virosomes by combining alphavirus replicon RNA comprising nucleic acid encoding and *gag* gene product or immunogenic fragment thereof, wherein the *gag* gene product or immunogenic fragment thereof is modified to inhibit formation of virus-like particles containing the *gag* gene product or the immunogenic fragment thereof and their release from a cell, alphavirus glycoproteins E1 and E2, non-cationic lipids and detergent; and

b) gradually removing detergent, whereby alphavirus replicon virosomes

producing a third population of alphavirus replicon virosomes by alphavirus replicon RNA comprising nucleic acid encoding the *pol* gene nonogenic fragment thereof, wherein the *pol* gene product or fragment thereof comprises a modification resulting in deletion or integrase, RNase H and reverse transcriptase functions in the *pol* gene nonogenic fragment thereof, alphavirus glycoproteins E1 and E2, non-and detergent; and

gradually removing detergent, whereby alphavirus replicon virosomes and

ing the first population of alphavirus replicon virosomes, the second alphavirus replicon virosomes and the third population of alphavirus virosomes to produce the population of alphavirus replicon virosomes of claim

iod of producing the population of alphavirus replicon virosomes of comprising:

producing a first population of alphavirus replicon virosomes by alphavirus replicon RNA comprising nucleic acid encoding and *env* gene nonogenic fragment thereof, alphavirus glycoproteins E1 and E2, non-and detergent; and

gradually removing detergent, whereby alphavirus replicon virosomes

producing a second population of alphavirus replicon virosomes by alphavirus replicon RNA comprising nucleic acid encoding and *gag* gene nonogenic fragment thereof, wherein the *gag* gene product or

immunogenic fragment thereof is modified to inhibit formation of virus-like particles containing the *gag* gene product or the immunogenic fragment thereof and their release from a cell, alphavirus glycoproteins E1 and E2, non-cationic lipids and detergent; and

b) gradually removing detergent, whereby alphavirus replicon virosomes are produced;

C) (a) producing a third population of alphavirus replicon virosomes by combining alphavirus replicon RNA comprising nucleic acid encoding the *pol* gene product or immunogenic fragment thereof, wherein the *pol* gene product or immunogenic fragment thereof comprises a modification resulting in inactivation of reverse transcriptase activity in the *pol* gene product or immunogenic fragment thereof, alphavirus glycoproteins E1 and E2, non-cationic lipids and detergent; and

b) gradually removing detergent, whereby alphavirus replicon virosomes are produced; and

D) combining the first population of alphavirus replicon virosomes, the second population of alphavirus replicon virosomes and the third population of alphavirus replicon virosomes to produce the population of alphavirus replicon virosomes of claim 48.

51. A method of eliciting an immune response in a subject, comprising administering to the subject an immunogenic amount of the composition of claim 47, in a pharmaceutically acceptable carrier.

52. A method of eliciting an immune response in a subject, comprising administering to the subject an immunogenic amount of the composition of claim 48, in a pharmaceutically acceptable carrier.

53. A method of treating or preventing infection by human immunodeficiency virus in a subject, comprising administering to the subject an immunogenic amount of the composition of claim 47, in a pharmaceutically acceptable carrier.

A method of treating or preventing infection by human immunodeficiency virus subject, comprising administering to the subject an immunogenic amount of the composition of claim 47, in a pharmaceutically acceptable carrier.

A composition comprising heparin affinity-purified alphavirus replicon particles, wherein the alphavirus replicon particles comprise at least one structural protein which comprises one or more attenuating mutations.

A method of preparing the heparin affinity-purified alphavirus particles of claim 55, comprising:

- a) producing alphavirus replicon particles, wherein the alphavirus replicon particles comprise at least one structural protein which comprises one or more attenuating mutations;
- b) loading the alphavirus replicon particles of step (a) in a heparin affinity chromatography column; and
- c) collecting the fraction from the column which contains the heparin affinity-purified alphavirus replicon particles.

A composition produced by the method of claim 56.

A method of producing VRP for use in a vaccine comprising:

- a) producing a plasmid encoding the nucleotide sequence of an alphavirus replicon RNA;
- b) producing a plasmid encoding the nucleotide sequence of one or more pre RNAs;
- c) transcribing the plasmids of steps (a) and (b) into RNA *in vitro*;
- d) electroporating the RNA of step (c) into a Vero cell line; and
- e) purifying VRP from the Vero cell line of step (d) by heparin affinity chromatography.

59. The method of claim 58, wherein the VRP is produced in large-scale.
60. VRP produced by the method of claim 59.
61. An isolated nucleic acid encoding a *pol* gene product or immunogenic fragment thereof of a human immunodeficiency virus, wherein the *pol* gene product or immunogenic fragment thereof comprises a modification resulting in deletion or inactivation of integrase, RNase H and reverse transcriptase functions in the *pol* gene product or immunogenic fragment thereof.
62. A composition comprising the nucleic acid of claim 61.
63. A vector comprising the nucleic acid of claim 61.
64. A cell comprising the vector of claim 63.
65. An alphavirus replicon particle comprising the nucleic acid of claim 61.
66. A method of making the alphavirus replicon particle of claim 65, comprising
 - a) providing a helper cell for producing an infectious, defective alphavirus particle, comprising in an alphavirus-permissive cell:
 - (i) an alphavirus replicon RNA, wherein the replicon RNA comprises an alphavirus packaging signal and a nucleic acid encoding a *pol* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, wherein the *pol* gene product or immunogenic fragment thereof comprises a modification resulting in deletion or inactivation of integrase, RNase H and reverse transcriptase functions in the *pol* gene product or immunogenic fragment thereof, and wherein the replicon RNA lacks sequences encoding alphavirus structural proteins;

(ii) a first helper RNA separate from said replicon RNA, said first helper RNA encoding at least one alphavirus structural protein and furthermore not encoding at least one other alphavirus structural protein; and

(iii) one or more additional helper RNA(s) separate from said replicon RNA and separate from said first helper RNA, said additional helper RNA(s) encoding at least one other alphavirus structural protein not encoded by said first helper RNA;

and with at least one of said helper RNAs lacking an alphavirus packaging signal;

wherein the combined expression of the alphavirus replicon RNA and the helper RNAs produces an assembled alphavirus particle which is able to infect a cell, and is unable to complete viral replication, and further wherein the population contains no detectable replication-competent alphavirus particles as determined by passage on permissive cells in culture;

- (b) producing the alphavirus particles in the helper cell; and
- (c) collecting the alphavirus particles from the helper cell.

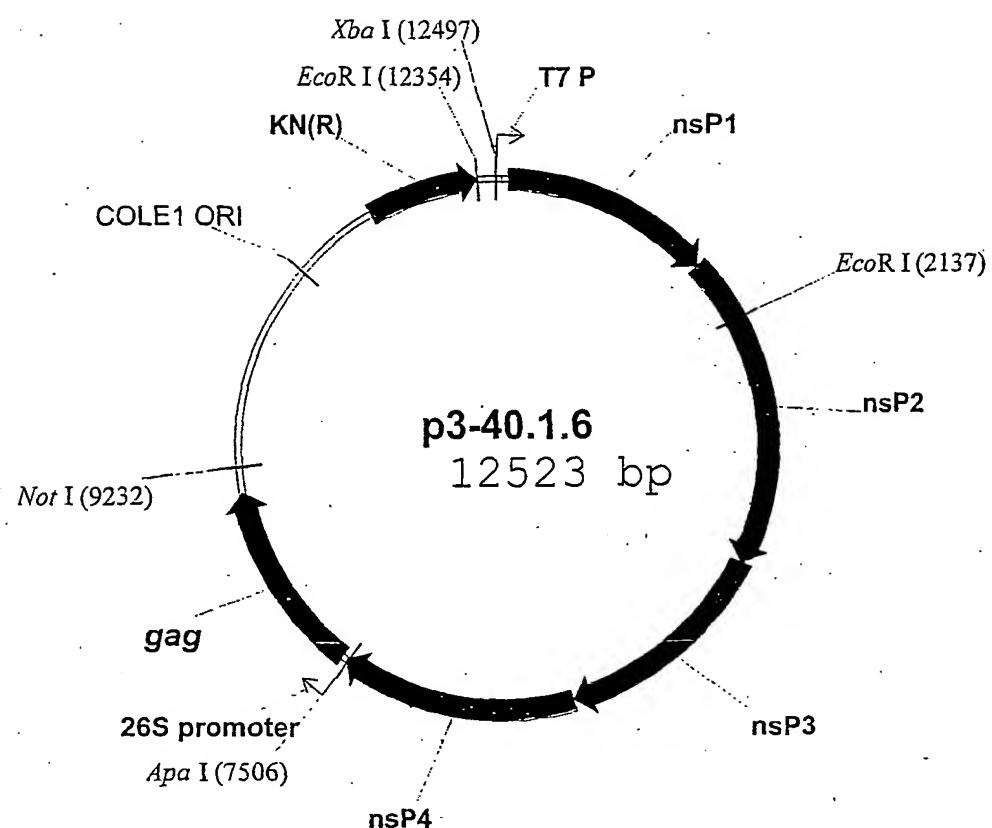
67. The method of claim 66, wherein at least one of said replicon RNA, said first helper RNA, and said one or more additional helper RNA(s) comprises one or more attenuating mutations.

68. An alphavirus replicon particle produced according to the method of claim 66.

69. An alphavirus replicon particle produced according to the method of claim 67.

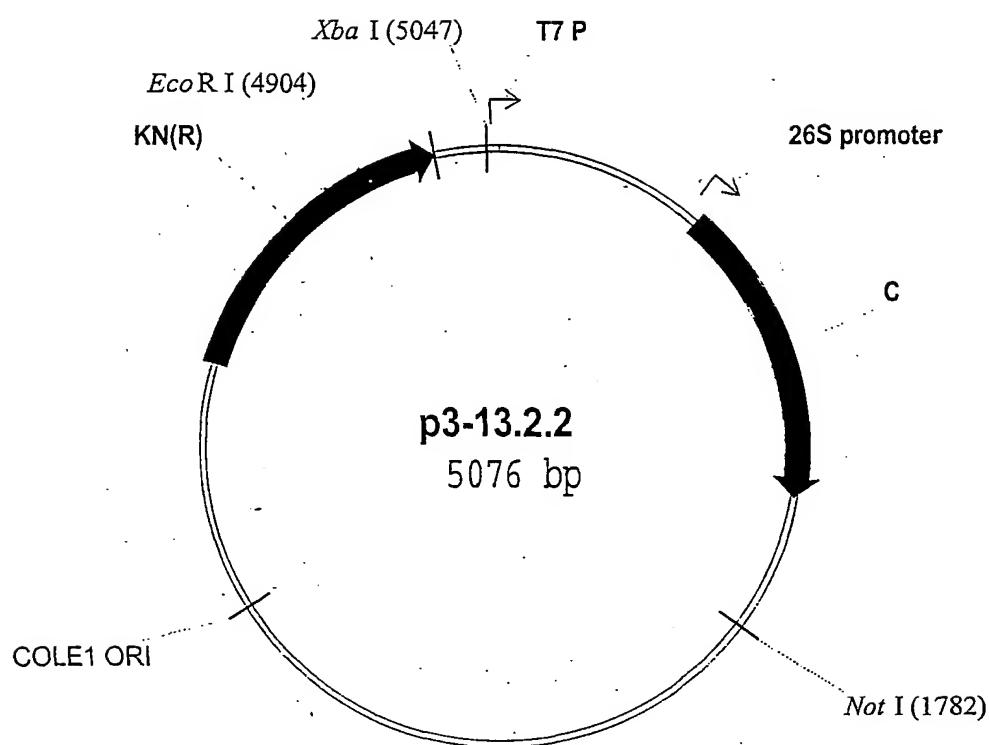
70. A method of inducing an immune response in a subject, comprising administering to the subject an immunogenic amount of the composition of claim 62 in a pharmaceutically acceptable carrier.

71. A method of inducing an immune response in a subject, comprising administering to the subject an immunogenic amount of the alphavirus replicon particle of claim 65 in a pharmaceutically acceptable carrier.
72. A method of treating or preventing infection by human immunodeficiency virus in a subject, comprising administering to the subject an immunogenic amount of the composition of claim 62 in a pharmaceutically acceptable carrier.
73. A method of treating or preventing infection by human immunodeficiency virus in a subject, comprising administering to the subject an immunogenic amount of the alphavirus replicon particle of claim 65 in a pharmaceutically acceptable carrier.
74. A method of inducing an immune response in a subject, comprising administering to the subject an immunogenic amount of a composition comprising the alphavirus replicon particles of claim 65 in a pharmaceutically acceptable carrier.
75. A method of treating or preventing infection by human immunodeficiency virus in a subject, comprising administering to the subject an immunogenic amount of a composition comprising the alphavirus replicon particles of claim 65 in a pharmaceutically acceptable carrier.

**FIG. 1**

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**FIG. 2**

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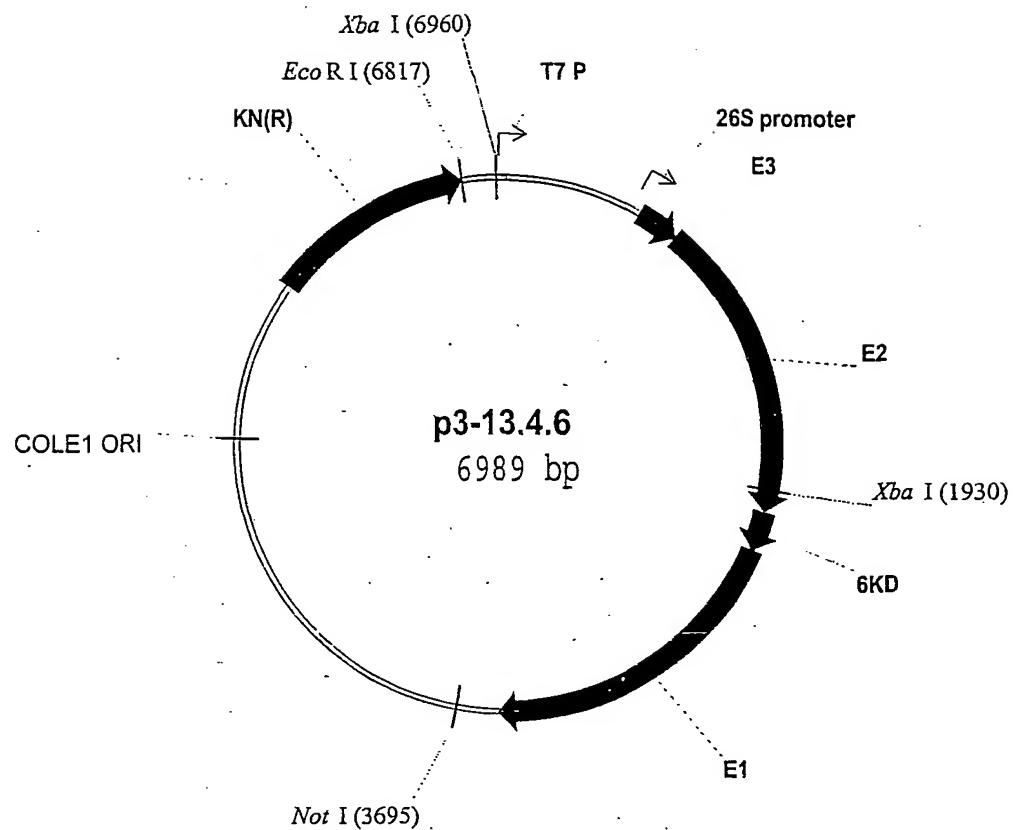
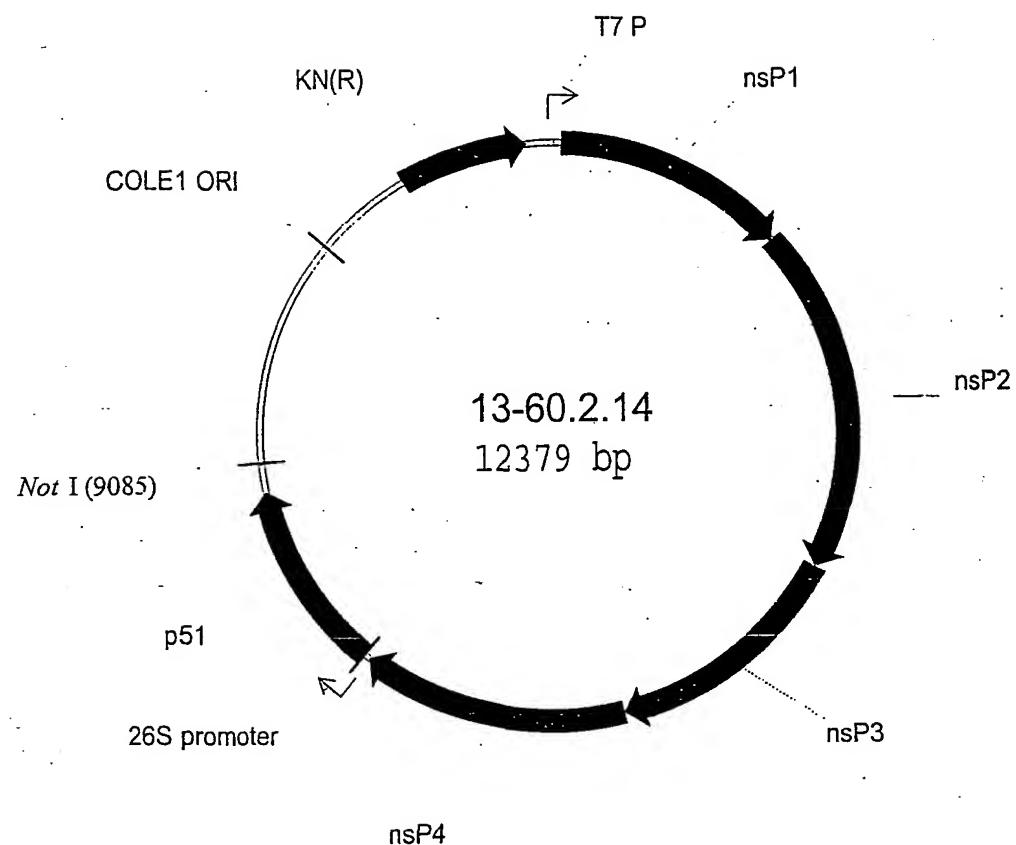


FIG. 3

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**FIG. 4**

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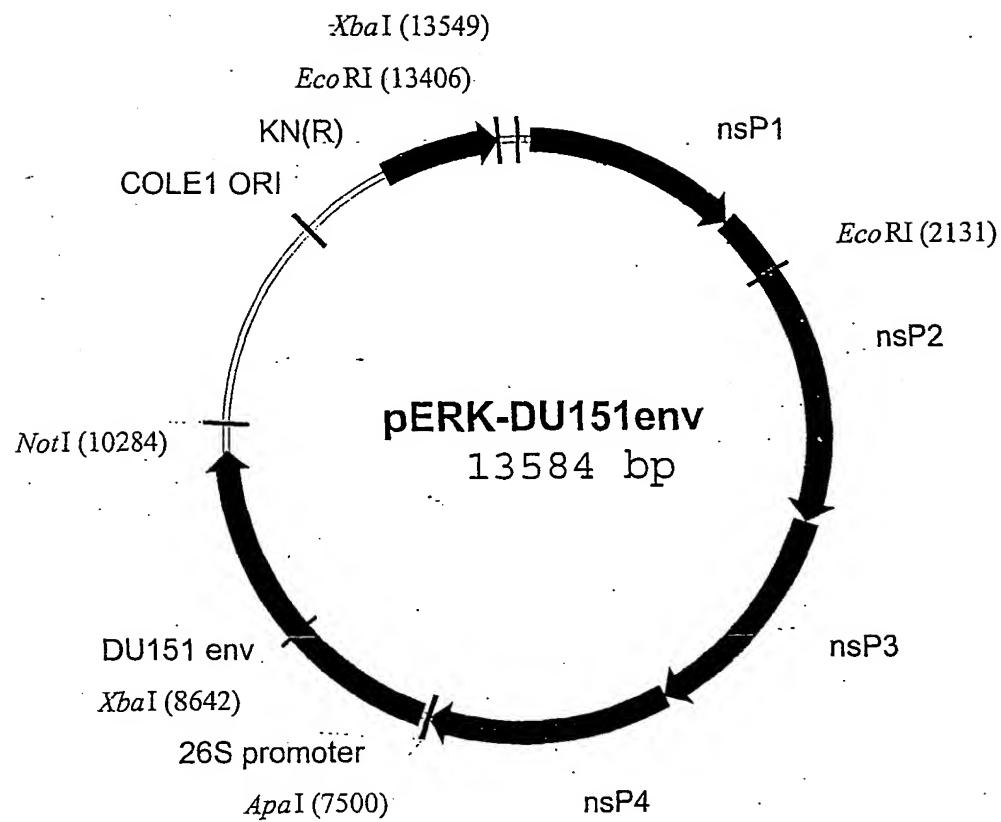


FIG. 5

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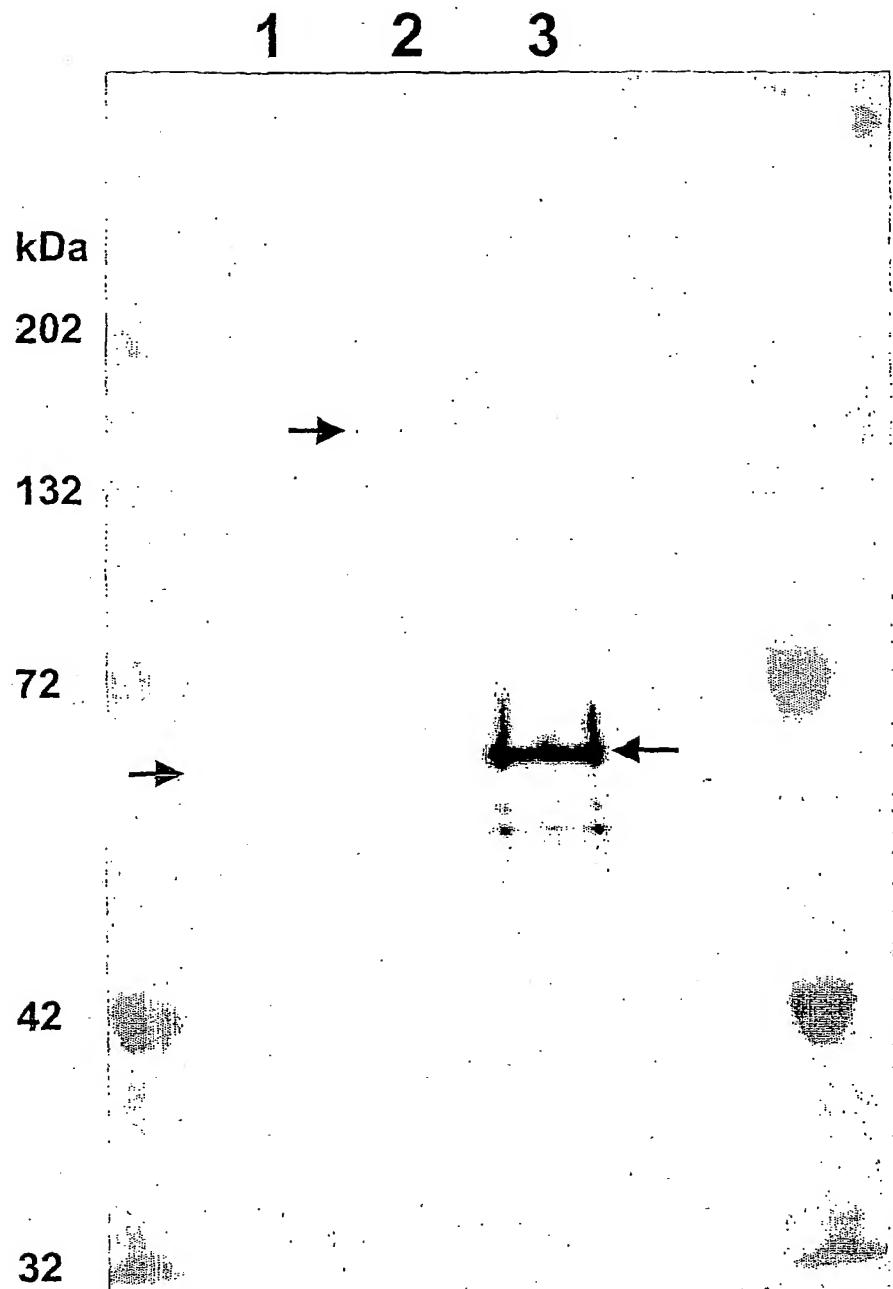
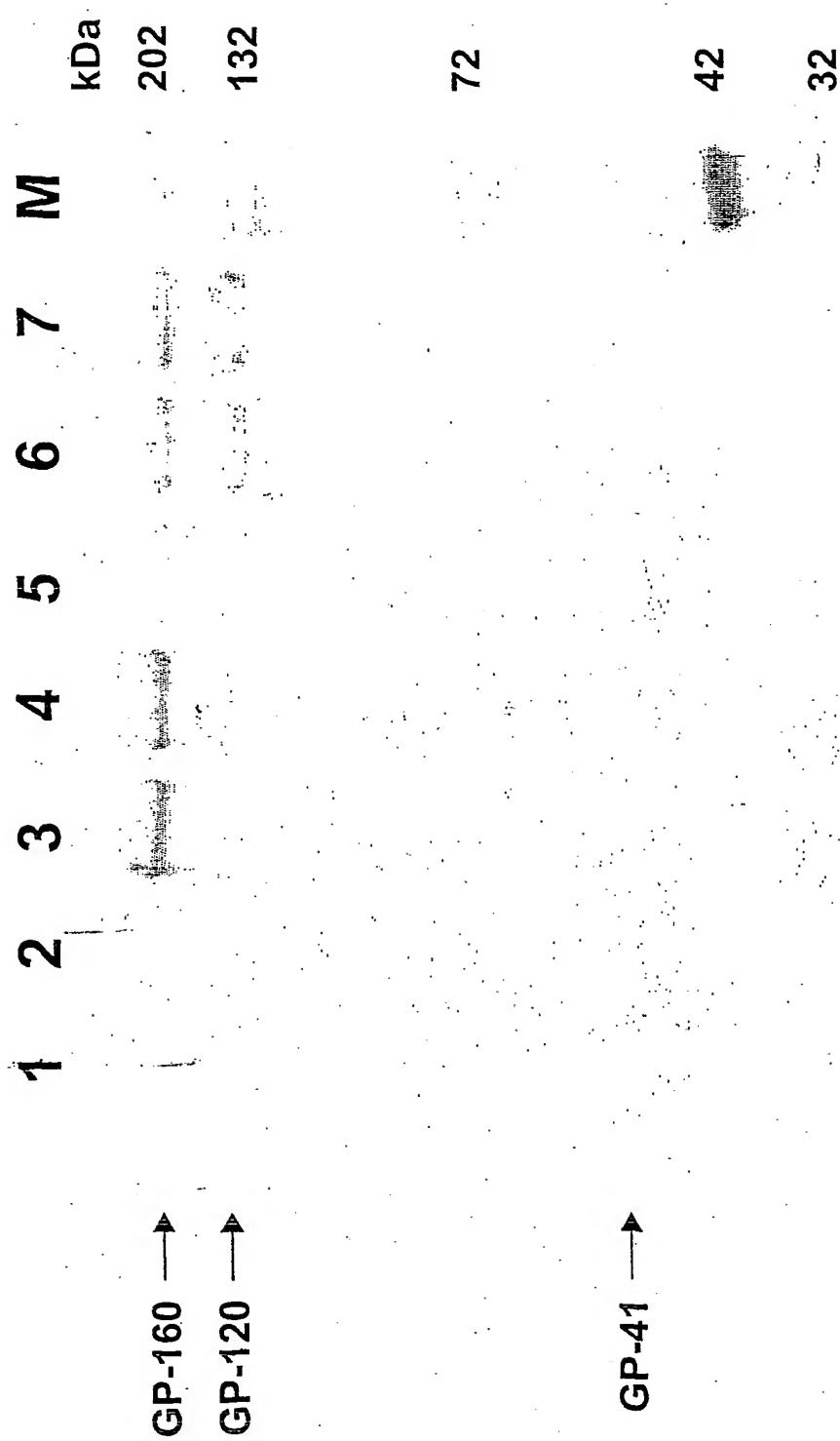


FIG. 6

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**FIG. 7**

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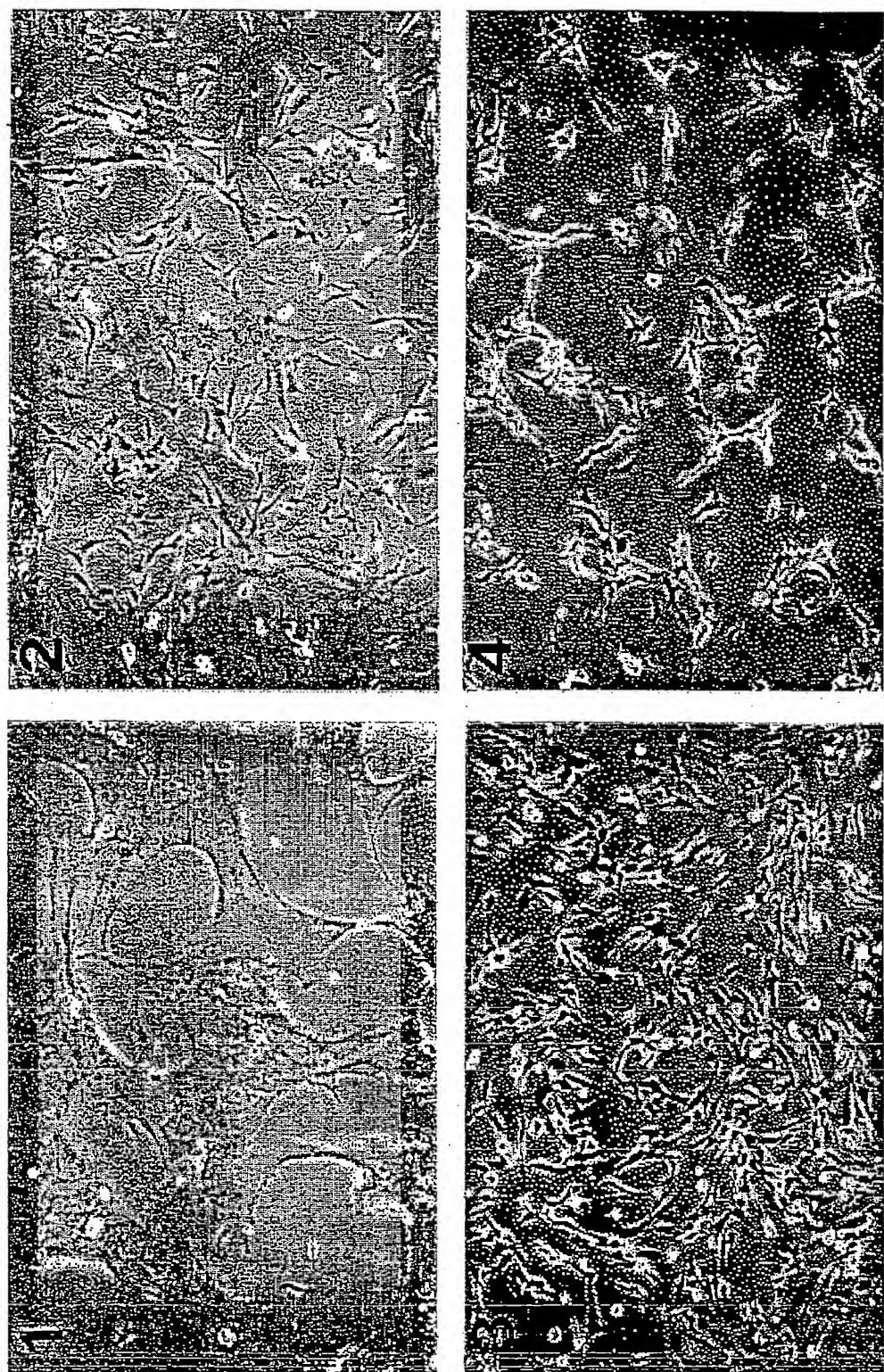
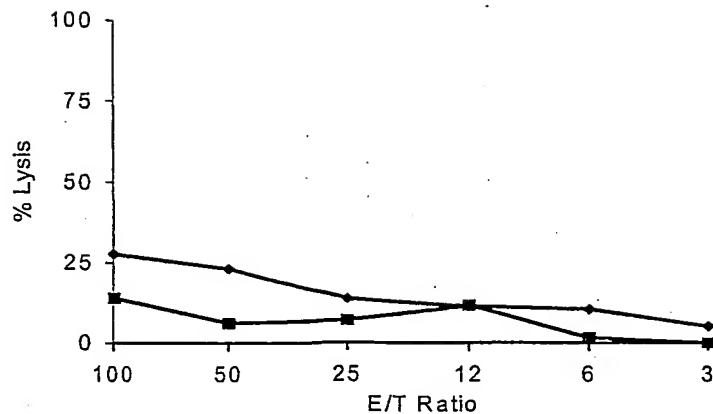
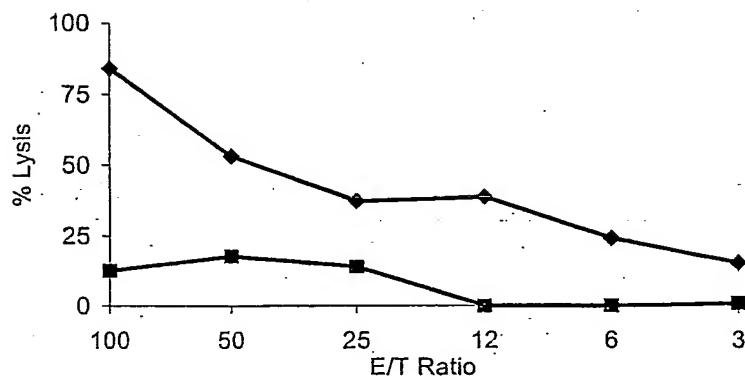
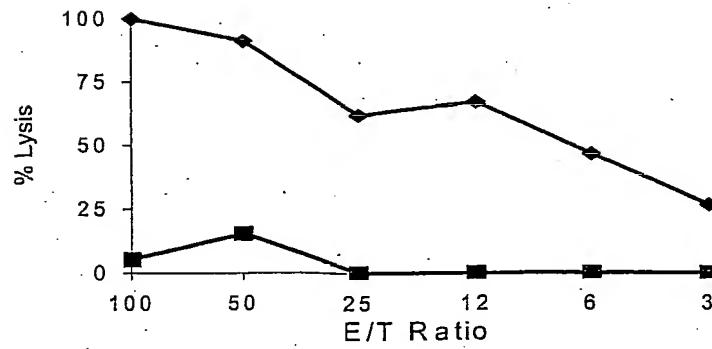


FIG. 8

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**FIG. 9A****FIG. 9B****FIG. 9C**

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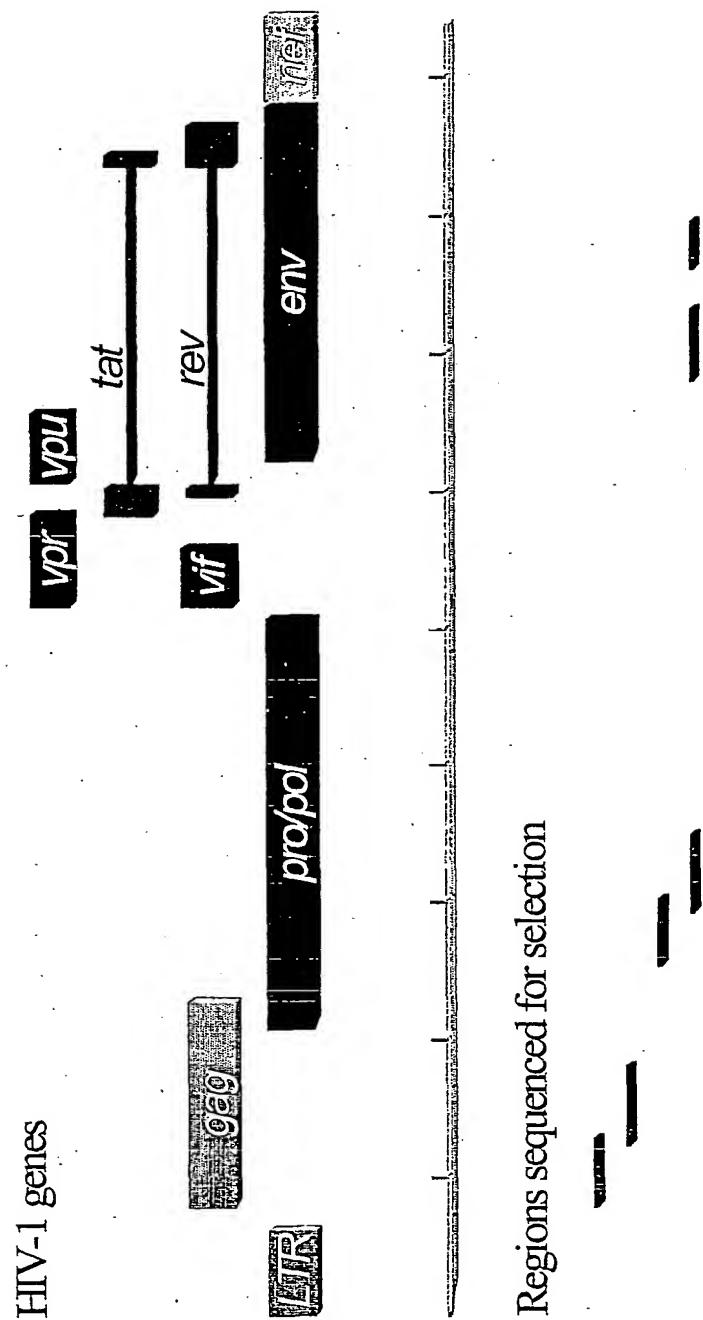


FIG. 10

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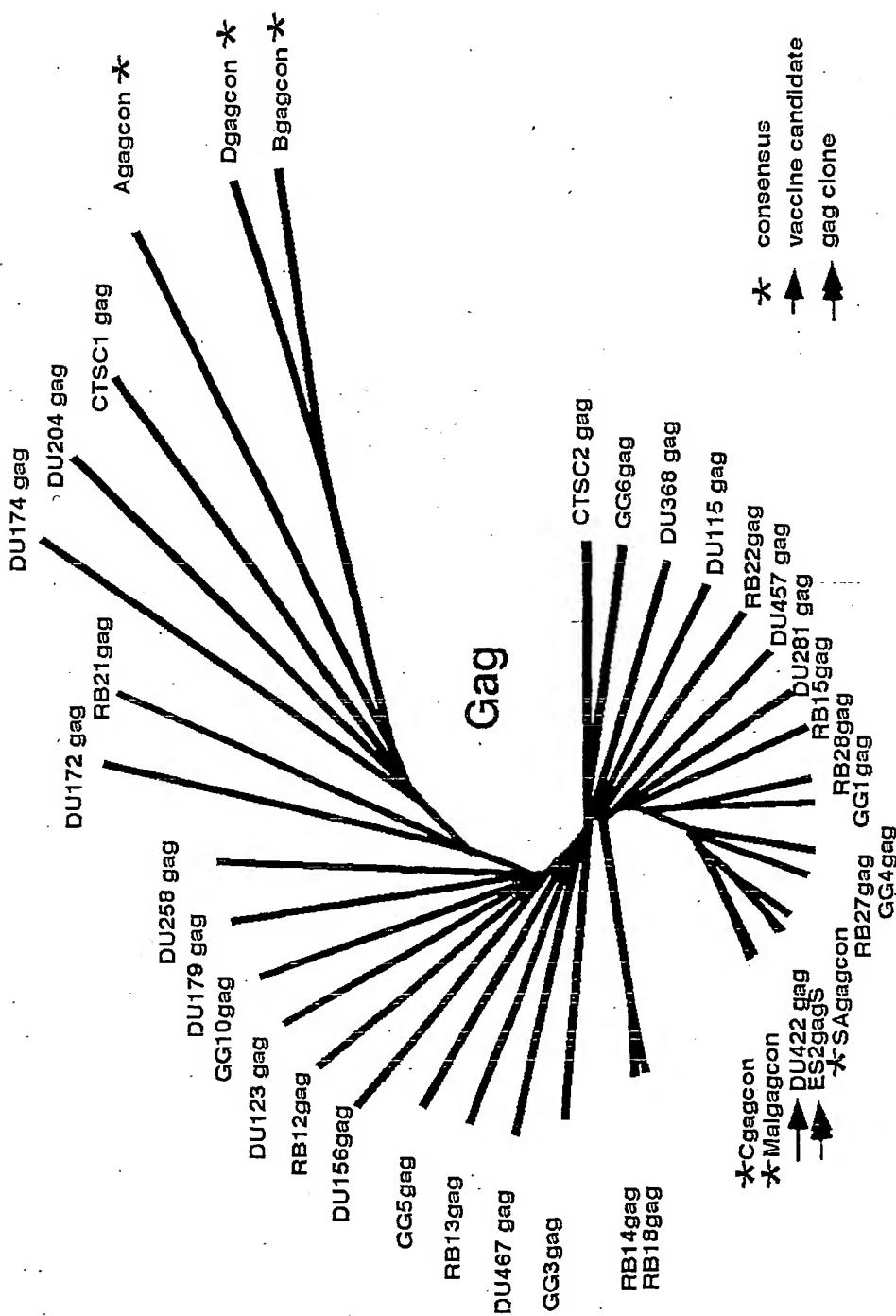


FIG. 11

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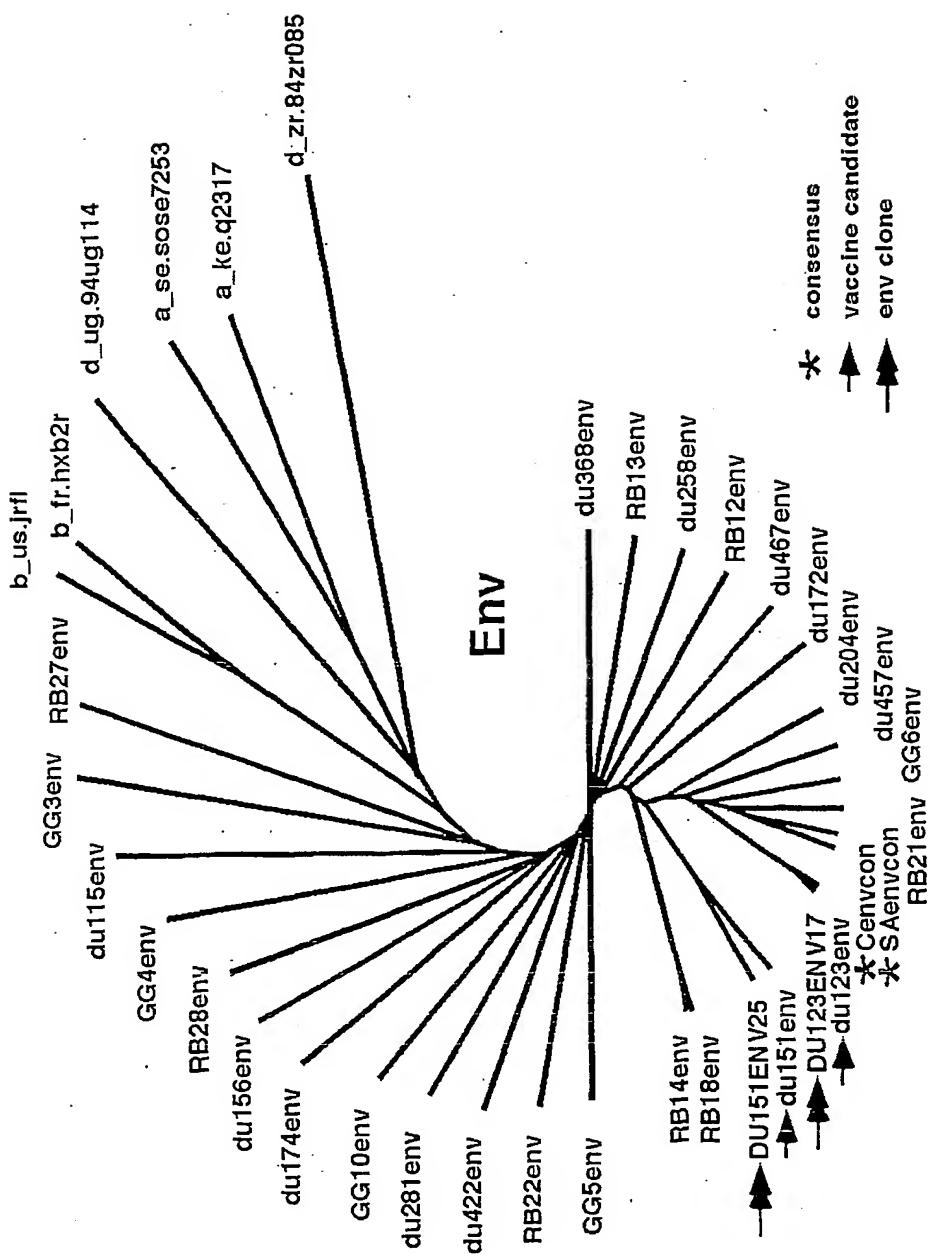


FIG. 12

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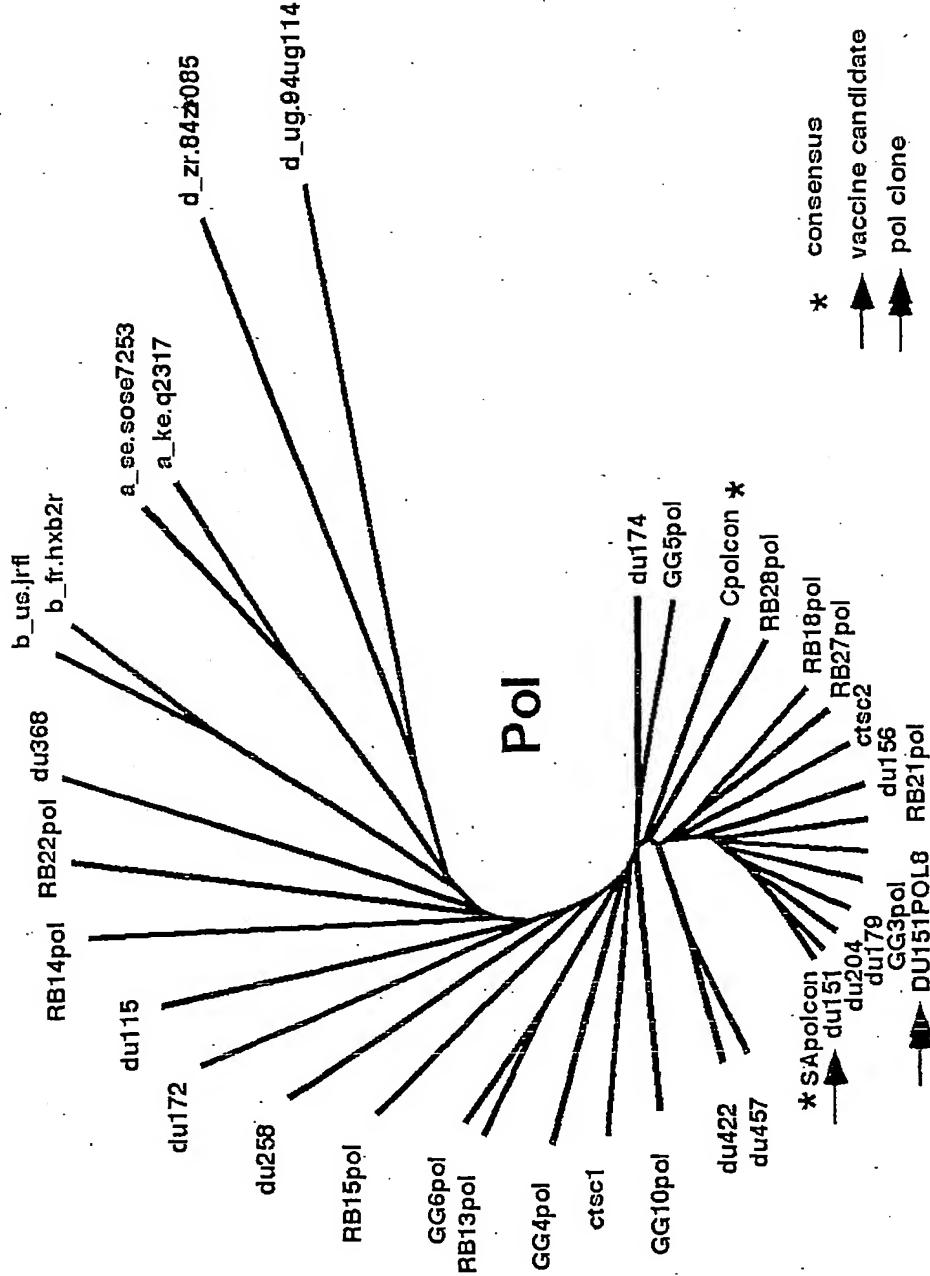


FIG. 13

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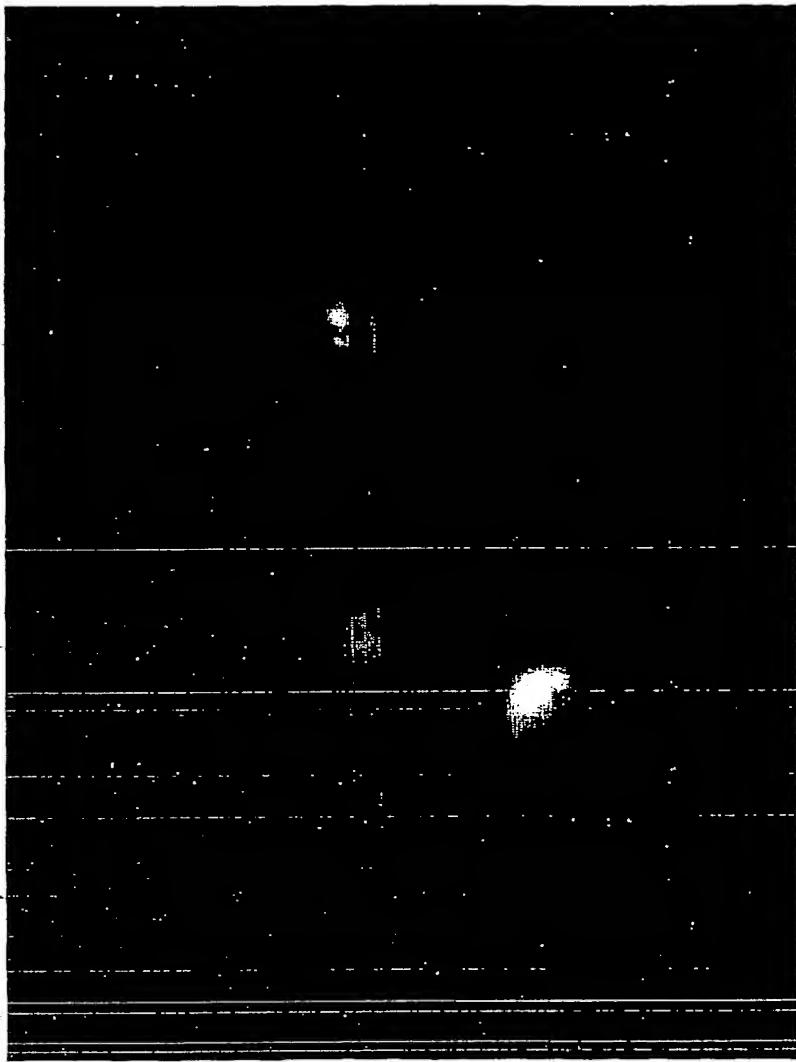


FIG. 14

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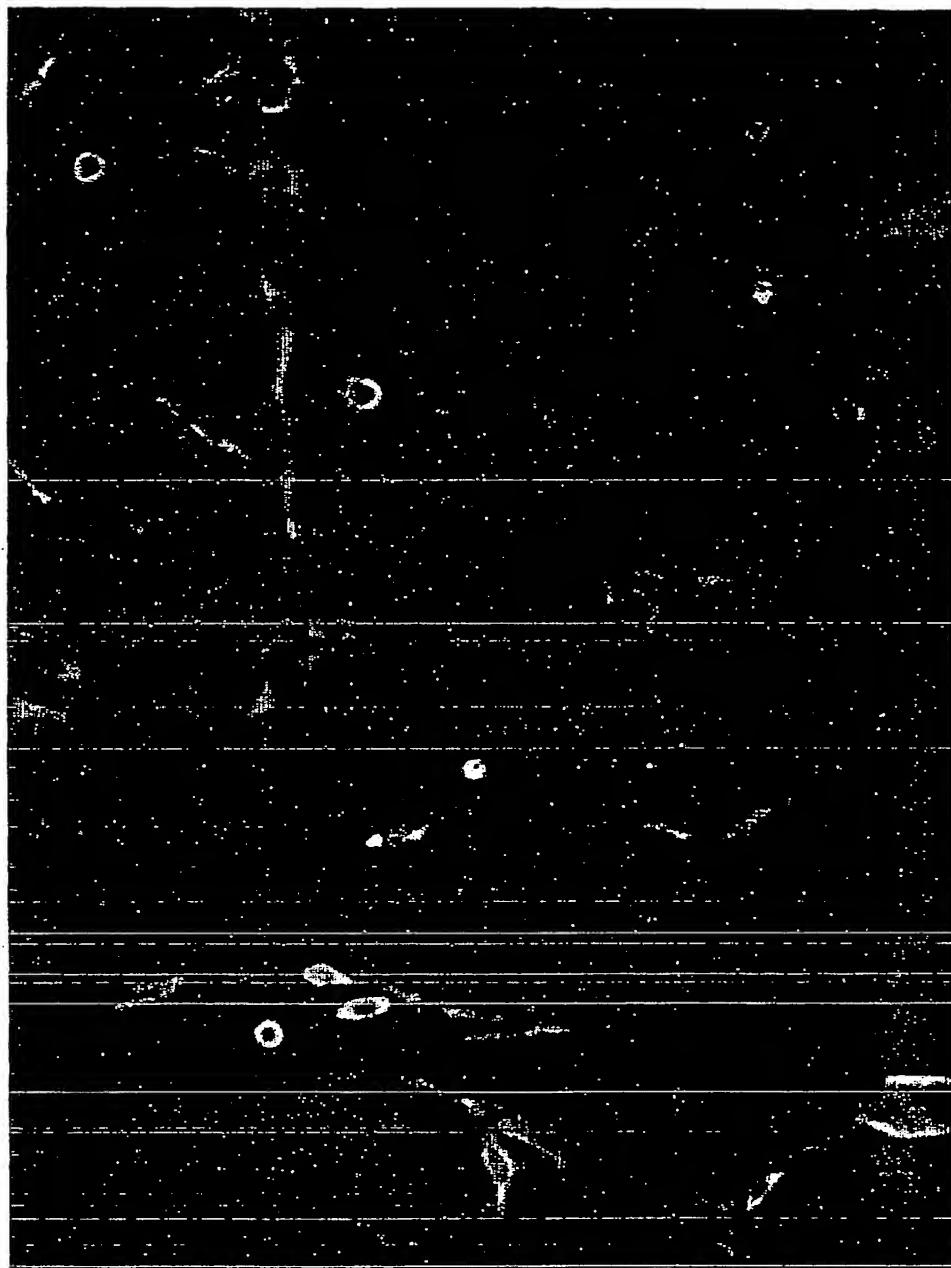


FIG. 15

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48

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Ala	Leu	Gln	Arg	Ser	Phe	Pro	Gln	Phe	Glu	Val	Glu	Ala	Lys	Gln	Val
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96

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144

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Gly His Ala Ile Pro Val Gln Asp Phe Gln Ala Leu Ser Glu Ser Ala	
610 615 620	
acc att gtg tac aac gaa cgt gag ttc gta aac agg tac ctg cac cat	1920
Thr Ile Val Tyr Asn Glu Arg Glu Phe Val Asn Arg Tyr Leu His His	
625 630 635 640	
att gcc aca cat gga gga gcg ctg aac act gat gaa gaa tat tac aaa	1968
Ile Ala Thr His Gly Gly Ala Leu Asn Thr Asp Glu Glu Tyr Tyr Lys	
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Thr Val Lys Pro Ser Glu His Asp Gly Glu Tyr Leu Tyr Asp Ile Asp	
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Arg Lys Gln Cys Val Lys Glu Leu Val Thr Gly Leu Gly Leu Thr	
675 680 685	
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Gly Glu Leu Val Asp Pro Pro Phe His Glu Phe Ala Tyr Glu Ser Leu	
690 695 700	
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Arg Thr Arg Pro Ala Ala Pro Tyr Gln Val Pro Thr Ile Gly Val Tyr	
705 710 715 720	
ggc gtg cca gga tca ggc aag tct ggc atc att aaa agc gca gtc acc	2208
Gly Val Pro Gly Ser Gly Lys Ser Gly Ile Ile Lys Ser Ala Val Thr	
725 730 735	

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tat att gac gaa gct ttt gct tgt cat gca ggt act ctc aga gcg ctc Tyr Ile Asp Glu Ala Phe Ala Cys His Ala Gly Thr Leu Arg Ala Leu 785 790 795 800	2400
ata gcc att ata aga cct aaa aag gca gtg ctc tgc ggg gat ccc aaa Ile Ala Ile Ile Arg Pro Lys Lys Ala Val Leu Cys Gly Asp Pro Lys 805 810 815	2448
cag tgc ggt ttt ttt aac atg atg tgc ctg aaa gtg cat ttt aac cac Gln Cys Gly Phe Phe Asn Met Met Cys Leu Lys Val His Phe Asn His 820 825 830	2496
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agt acc aaa cct aag cag gac gat ctc att ctc act tgt ttc aga ggg Ser Thr Lys Pro Lys Gln Asp Asp Leu Ile Leu Thr Cys Phe Arg Gly 885 890 895	2688
tgg gtg aag cag ttg caa ata gat tac aaa ggc aac gaa ata atg acg Trp Val Lys Gln Leu Gln Ile Asp Tyr Lys Gly Asn Glu Ile Met Thr 900 905 910	2736
gca gct gcc tct caa ggg ctg acc cgt aaa ggt gtg tat gcc gtt cgg Ala Ala Ala Ser Gln Gly Leu Thr Arg Lys Gly Val Tyr Ala Val Arg 915 920 925	2784
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aac gtc cta ctg acc cgc acg gag gac cgc atc gtg tgg aaa aca cta Asn Val Leu Leu Thr Arg Thr Glu Asp Arg Ile Val Trp Lys Thr Leu 945 950 955 960	2880

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 Phe Thr Ala Thr Ile Glu Glu Trp Gln Ala Glu His Asp Ala Ile Met
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agg cac atc ttg gag aga ccg gac cct acc gac gtc ttc cag aat aag 3024
 Arg His Ile Leu Glu Arg Pro Asp Pro Thr Asp Val Phe Gln Asn Lys
 995 1000 1005

gca aac gtg tgt tgg gcc aag gct tta gtg ccg gtg ctg aag acc gct 3072
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 Gly Ile Asp Met Thr Thr Glu Gln Trp Asn Thr Val Asp Tyr Phe Glu
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 Thr Asp Lys Ala His Ser Ala Glu Ile Val Leu Asn Gln Leu Cys Val
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 Arg Phe Phe Gly Leu Asp Leu Asp Ser Gly Leu Phe Ser Ala Pro Thr
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 Val Pro Leu Ser Ile Arg Asn Asn His Trp Asp Asn Ser Pro Ser Pro
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 Tyr Pro Gln Leu Pro Arg Ala Val Ala Thr Gly Arg Val Tyr Asp Met
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aac act ggt aca ctg cgc aat tat gat ccg cgc ata aac cta gta cct 3408
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 1125 1130 1135

gta aac aga aga ctg cct cat gct tta gtc ctc cac cat aat gaa cac 3456
 Val Asn Arg Arg Leu Pro His Ala Leu Val Leu His His Asn Glu His
 1140 1145 1150

cca cag agt gac ttt tct tca ttc gtc agc aaa ttg aag ggc aga act 3504
 Pro Gln Ser Asp Phe Ser Ser Phe Val Ser Lys Leu Lys Gly Arg Thr
 1155 1160 1165

gtc ctg gtg gtc ggg gaa aag ttg tcc gtc cca ggc aaa atg gtt gac 3552
 Val Leu Val Val Gly Glu Lys Leu Ser Val Pro Gly Lys Met Val Asp
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cc cca tat aaa tac cat cac tat cag cag tgt gaa gac cat gcc ar Pro Tyr Lys Tyr His His Tyr Gln Gln Cys Glu Asp His Ala 1220 1225 1230	3696
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ga acc tgt gtc agc ata ggt tat ggt tac gct gac agg gcc agc ly Thr Cys Val Ser Ile Gly Tyr Gly Tyr Ala Asp Arg Ala Ser 250 1255 1260	3792
gc atc att ggt gct ata gcg cgg cag ttc aag ttt tcc cgg gta er Ile Ile Gly Ala Ile Ala Arg Gln Phe Lys Phe Ser Arg Val 1270 1275 1280	3840
aa ccg aaa tcc tca ctt gaa gag acg gaa gtt ctg ttt gta ttc ys Pro Lys Ser Ser Leu Glu Glu Thr Glu Val Leu Phe Val Phe 1285 1290 1295	3888
gg tac gat cgc aag gcc cgt acg cac aat cct tac aag ctt tca ly Tyr Asp Arg Lys Ala Arg Thr His Asn Pro Tyr Lys Leu Ser 1300 1305 1310	3936
cc ttg acc aac att tat aca ggt tcc aga ctc cac gaa gcc gga hr Leu Thr Asn Ile Tyr Thr Gly Ser Arg Leu His Glu Ala Gly 1315 1320 1325	3984
ca ccc tca tat cat gtg gtg cga ggg gat att gcc acg gcc acc la Pro Ser Tyr His Val Val Arg Gly Asp Ile Ala Thr Ala Thr 330 1335 1340	4032
ga gtg att ata aat gct gct aac agc aaa gga caa cct ggc gga ly Val Ile Ile Asn Ala Ala Asn Ser Lys Gly Gln Pro Gly Gly 1350 1355 1360	4080
tg tgc gga gcg ctg tat aag aag ttc ccg gaa agc ttc gat tta al Cys Gly Ala Leu Tyr Lys Lys Phe Pro Glu Ser Phe Asp Leu 1365 1370 1375	4128
cg atc gaa gta gga aaa gcg cga ctg gtc aaa ggt gca gct aaa ro Ile Glu Val Gly Lys Ala Arg Leu Val Lys Gly Ala Ala Lys 1380 1385 1390	4176
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gtc aac gat aac aat tac aag tca gtc att cca ctg ttg tcc acc 4320
 Val Asn Asp Asn Asn Tyr Lys Ser Val Ala Ile Pro Leu Leu Ser Thr
 1425 1430 1435 1440

ggc atc ttt tcc ggg aac aaa gat cga cta acc caa tca ttg aac cat 4368
 Gly Ile Phe Ser Gly Asn Lys Asp Arg Leu Thr Gln Ser Leu Asn His
 1445 1450 1455

ttg ctg aca gct tta gac acc act gat gca gat gta gcc ata tac tgc 4416
 Leu Leu Thr Ala Leu Asp Thr Asp Ala Asp Val Ala Ile Tyr Cys
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agg gac aag aaa tgg gaa atg act ctc aag gaa gca gtg gct agg aga 4464
 Arg Asp Lys Lys Trp Glu Met Thr Leu Lys Glu Ala Val Ala Arg Arg
 1475 1480 1485

gaa gca gtg gag gag ata tgc ata tcc gac gac tct tca gtg aca gaa 4512
 Glu Ala Val Glu Glu Ile Cys Ile Ser Asp Asp Ser Ser Val Thr Glu
 1490 1495 1500

cct gat gca gag ctg gtg agg gtg cat ccg aag agt tct ttg gct gga 4560
 Pro Asp Ala Glu Leu Val Arg Val His Pro Lys Ser Ser Leu Ala Gly
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 Gly Thr Lys Phe His Gln Ala Ala Lys Asp Ile Ala Glu Ile Asn Ala
 1540 1545 1550

atg tgg ccc gtt gca acg gag gcc aat gag cag gta tgc atg tat atc 4704
 Met Trp Pro Val Ala Thr Glu Ala Asn Glu Gln Val Cys Met Tyr Ile
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ctc gga gaa agc atg agc agt att agg tcg aaa tgc ccc gtc gaa gag 4752
 Leu Gly Glu Ser Met Ser Ser Ile Arg Ser Lys Cys Pro Val Glu Glu
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tcg gaa gcc tcc aca cca cct agc acg ctg cct tgc ttg tgc atc cat 4800
 Ser Glu Ala Ser Thr Pro Pro Ser Thr Leu Pro Cys Leu Cys Ile His
 1585 1590 1595 1600

gcc atg act cca gaa aga gta cag cgc cta aaa gcc tca cgt cca gaa 4848
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ccg cca ggc gtg aat agg gtg atc act aga gag gag ctc gag ggc ctt Pro Pro Gly Val Asn Arg Val Ile Thr Arg Glu Glu Leu Ala Leu 1825 1830 1835 1840	5520
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gcg ttc gta gca caa caa tga cgg ttt gat gcg ggt gca tac atc	5664
Ala Phe Val Ala Gln Gln Gln * Arg Phe Asp Ala Gly Ala Tyr Ile	
1875 1880 1885	
ttt tcc tcc gac acc ggt caa ggg cat tta caa caa aaa tca gta agg	5712
Phe Ser Ser Asp Thr Gly Gln Gly His Leu Gln Gln Lys Ser Val Arg	
1890 1895 1900	
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Gln Thr Val Leu Ser Glu Val Val Leu Glu Arg Thr Glu Leu Glu Ile	
1905 1910 1915	
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Ser Tyr Ala Pro Arg Leu Asp Gln Glu Lys Glu Glu Leu Leu Arg Lys	
1920 1925 1930 1935	
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Lys Leu Gln Leu Asn Pro Thr Pro Ala Asn Arg Ser Arg Tyr Gln Ser	
1940 1945 1950	
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Arg Lys Val Glu Asn Met Lys Ala Ile Thr Ala Arg Arg Ile Leu Gln	
1955 1960 1965	
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Gly Leu Gly His Tyr Leu Lys Ala Glu Gly Lys Val Glu Cys Tyr Arg	
1970 1975 1980	
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Thr Leu His Pro Val Pro Leu Tyr Ser Ser Ser Val Asn Arg Ala Phe	
1985 1990 1995	
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Ser Ser Pro Lys Val Ala Val Glu Ala Cys Asn Ala Met Leu Lys Glu	
2000 2005 2010 2015	
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Asn Phe Pro Thr Val Ala Ser Tyr Cys Ile Ile Pro Glu Tyr Asp Ala	
2020 2025 2030	
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Tyr Leu Asp Met Val Asp Gly Ala Ser Cys Cys Leu Asp Thr Ala Ser	
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Phe Cys Pro Ala Lys Leu Arg Ser Phe Pro Lys Lys His Ser Tyr Leu	
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 2115 2120 2125

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 2145 2150 2155

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Arg Glu Arg Leu Thr Gly Ser Pro Cys Ala Ala Phe Ile Gly Asp Asp	
2340 2345 2350	
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<211> 2492

<212> PRT

<213> Artificial Sequence

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<220>

<223> Description of Artificial Sequence; Note =
synthetic construct

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Thr	Asp	Asn	Asp	His	Ala	Asn	Ala	Arg	Ala	Phe	Ser	His	Leu	Ala	Ser
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Lys	Leu	Ile	Glu	Thr	Glu	Val	Asp	Pro	Ser	Asp	Thr	Ile	Leu	Asp	Ile
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Gly	Ser	Ala	Pro	Ala	Arg	Arg	Met	Tyr	Ser	Lys	His	Lys	Tyr	His	Cys
					65			70			75		80		
Ile	Cys	Pro	Met	Arg	Cys	Ala	Glu	Asp	Pro	Asp	Arg	Leu	Tyr	Lys	Tyr
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Ala	Thr	Lys	Leu	Lys	Lys	Asn	Cys	Lys	Glu	Ile	Thr	Asp	Lys	Glu	Leu
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Asp	Lys	Lys	Met	Lys	Glu	Leu	Ala	Ala	Val	Met	Ser	Asp	Pro	Asp	Leu
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Glu	Thr	Glu	Thr	Met	Cys	Leu	His	Asp	Asp	Glu	Ser	Cys	Arg	Tyr	Glu
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Gly	Gln	Val	Ala	Val	Tyr	Gln	Asp	Val	Tyr	Ala	Val	Asp	Gly	Pro	Thr
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Ser	Leu	Tyr	His	Gln	Ala	Asn	Lys	Gly	Val	Arg	Val	Ala	Tyr	Trp	Ile
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Gly	Phe	Asp	Thr	Thr	Pro	Phe	Met	Phe	Lys	Asn	Leu	Ala	Gly	Ala	Tyr
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Pro	Ser	Tyr	Ser	Thr	Asn	Trp	Ala	Asp	Glu	Thr	Val	Leu	Thr	Ala	Arg
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Asn	Ile	Gly	Leu	Cys	Ser	Ser	Asp	Val	Met	Glu	Arg	Ser	Arg	Arg	Gly
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Met	Ser	Ile	Leu	Arg	Lys	Tyr	Leu	Lys	Pro	Ser	Asn	Asn	Val	Leu	
					225			230				235		240	
Phe	Ser	Val	Gly	Ser	Thr	Ile	Tyr	His	Glu	Lys	Arg	Asp	Leu	Leu	Arg
					245			250				255			
Ser	Trp	His	Leu	Pro	Ser	Val	Phe	His	Leu	Arg	Gly	Lys	Gln	Asn	Tyr
					260			265				270			
Thr	Cys	Arg	Cys	Glu	Thr	Ile	Val	Ser	Cys	Asp	Gly	Tyr	Val	Val	Lys
					275			280				285			
Arg	Ile	Ala	Ile	Ser	Pro	Gly	Leu	Tyr	Gly	Lys	Pro	Ser	Gly	Tyr	Ala
					290			295				300			
Ala	Thr	Met	His	Arg	Glu	Gly	Phe	Leu	Cys	Cys	Lys	Val	Thr	Asp	Thr
					305			310				315		320	
Leu	Asn	Gly	Glu	Arg	Val	Ser	Phe	Pro	Val	Cys	Thr	Tyr	Val	Pro	Ala
					325			330				335			
Thr	Leu	Cys	Asp	Gln	Met	Thr	Gly	Ile	Leu	Ala	Thr	Asp	Val	Ser	Ala
					340			345				350			
Asp	Asp	Ala	Gln	Lys	Leu	Leu	Val	Gly	Leu	Asn	Gln	Arg	Ile	Val	Val
					355			360				365			
Asn	Gly	Arg	Thr	Gln	Arg	Asn	Thr	Asn	Thr	Met	Lys	Asn	Tyr	Leu	Leu
					370			375				380			
Pro	Val	Val	Ala	Gln	Ala	Phe	Ala	Arg	Trp	Ala	Lys	Glu	Tyr	Lys	Glu
					385			390				395		400	

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Asp Gln Glu Asp Glu Arg Pro Leu Gly Leu Arg Asp Arg Gln Leu Val
 405 410 415
 Met Gly Cys Cys Trp Ala Phe Arg Arg His Lys Ile Thr Ser Ile Tyr
 420 425 430
 Lys Arg Pro Asp Thr Gln Thr Ile Ile Lys Val Asn Ser Asp Phe His
 435 440 445
 Ser Phe Val Leu Pro Arg Ile Gly Ser Asn Thr Leu Glu Ile Gly Leu
 450 455 460
 Arg Thr Arg Ile Arg Lys Met Leu Glu Glu His Lys Glu Pro Ser Pro
 465 470 475 480
 Leu Ile Thr Ala Glu Asp Val Gln Glu Ala Lys Cys Ala Ala Asp Glu
 485 490 495
 Ala Lys Glu Val Arg Glu Ala Glu Leu Arg Ala Ala Leu Pro Pro
 500 505 510
 Leu Ala Ala Asp Val Glu Glu Pro Thr Leu Glu Ala Asp Val Asp Leu
 515 520 525
 Met Leu Gln Glu Ala Gly Ala Gly Ser Val Glu Thr Pro Arg Gly Leu
 530 535 540
 Ile Lys Val Thr Ser Tyr Ala Gly Glu Asp Lys Ile Gly Ser Tyr Ala
 545 550 555 560
 Val Leu Ser Pro Gln Ala Val Leu Lys Ser Glu Lys Leu Ser Cys Ile
 565 570 575
 His Pro Leu Ala Glu Gln Val Ile Val Ile Thr His Ser Gly Arg Lys
 580 585 590
 Gly Arg Tyr Ala Val Glu Pro Tyr His Gly Lys Val Val Val Pro Glu
 595 600 605
 Gly His Ala Ile Pro Val Gln Asp Phe Gln Ala Leu Ser Glu Ser Ala
 610 615 620
 Thr Ile Val Tyr Asn Glu Arg Glu Phe Val Asn Arg Tyr Leu His His
 625 630 635 640
 Ile Ala Thr His Gly Gly Ala Leu Asn Thr Asp Glu Glu Tyr Tyr Lys
 645 650 655
 Thr Val Lys Pro Ser Glu His Asp Gly Glu Tyr Leu Tyr Asp Ile Asp
 660 665 670
 Arg Lys Gln Cys Val Lys Lys Glu Leu Val Thr Gly Leu Gly Leu Thr
 675 680 685
 Gly Glu Leu Val Asp Pro Pro Phe His Glu Phe Ala Tyr Glu Ser Leu
 690 695 700
 Arg Thr Arg Pro Ala Ala Pro Tyr Gln Val Pro Thr Ile Gly Val Tyr
 705 710 715 720
 Gly Val Pro Gly Ser Gly Lys Ser Gly Ile Ile Lys Ser Ala Val Thr
 725 730 735
 Lys Lys Asp Leu Val Val Ser Ala Lys Lys Glu Asn Cys Ala Glu Ile
 740 745 750
 Ile Arg Asp Val Lys Lys Met Lys Gly Leu Asp Val Asn Ala Arg Thr
 755 760 765
 Val Asp Ser Val Leu Leu Asn Gly Cys Lys His Pro Val Glu Thr Leu
 770 775 780
 Tyr Ile Asp Glu Ala Phe Ala Cys His Ala Gly Thr Leu Arg Ala Leu
 785 790 795 800
 Ile Ala Ile Ile Arg Pro Lys Lys Ala Val Leu Cys Gly Asp Pro Lys
 805 810 815
 Gln Cys Gly Phe Phe Asn Met Met Cys Leu Lys Val His Phe Asn His
 820 825 830

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Glu Ile Cys Thr Gln Val Phe His Lys Ser Ile Ser Arg Arg Cys Thr
 835 840 845
 Lys Ser Val Thr Ser Val Val Ser Thr Leu Phe Tyr Asp Lys Lys Met
 850 855 860
 Arg Thr Thr Asn Pro Lys Glu Thr Lys Ile Val Ile Asp Thr Thr Gly
 865 870 875 880
 Ser Thr Lys Pro Lys Gln Asp Asp Leu Ile Leu Thr Cys Phe Arg Gly
 885 890 895
 Trp Val Lys Gln Leu Gln Ile Asp Tyr Lys Gly Asn Glu Ile Met Thr
 900 905 910
 Ala Ala Ala Ser Gln Gly Leu Thr Arg Lys Gly Val Tyr Ala Val Arg
 915 920 925
 Tyr Lys Val Asn Glu Asn Pro Leu Tyr Ala Pro Thr Ser Glu His Val
 930 935 940
 Asn Val Leu Leu Thr Arg Thr Glu Asp Arg Ile Val Trp Lys Thr Leu
 945 950 955 960
 Ala Gly Asp Pro Trp Ile Lys Thr Leu Thr Ala Lys Tyr Pro Gly Asn
 965 970 975
 Phe Thr Ala Thr Ile Glu Glu Trp Gln Ala Glu His Asp Ala Ile Met
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 Arg His Ile Leu Glu Arg Pro Asp Pro Thr Asp Val Phe Gln Asn Lys
 995 1000 1005
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 Gly Ile Asp Met Thr Thr Glu Gln Trp Asn Thr Val Asp Tyr Phe Glu
 1025 1030 1035 1040
 Thr Asp Lys Ala His Ser Ala Glu Ile Val Leu Asn Gln Leu Cys Val
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 1090 1095 1100
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 Asn Thr Gly Thr Leu Arg Asn Tyr Asp Pro Arg Ile Asn Leu Val Pro
 1125 1130 1135
 Val Asn Arg Arg Leu Pro His Ala Leu Val Leu His His Asn Glu His
 1140 1145 1150
 Pro Gln Ser Asp Phe Ser Ser Phe Val Ser Lys Leu Lys Gly Arg Thr
 1155 1160 1165
 Val Leu Val Val Gly Glu Lys Leu Ser Val Pro Gly Lys Met Val Asp
 1170 1175 1180
 Trp Leu Ser Asp Arg Pro Glu Ala Thr Phe Arg Ala Arg Leu Asp Leu
 1185 1190 1195 1200
 Gly Ile Pro Gly Asp Val Pro Lys Tyr Asp Ile Ile Phe Val Asn Val
 1205 1210 1215
 Arg Thr Pro Tyr Lys Tyr His His Tyr Gln Gln Cys Glu Asp His Ala
 1220 1225 1230
 Ile Lys Leu Ser Met Leu Thr Lys Lys Ala Cys Leu His Leu Asn Pro
 1235 1240 1245
 Gly Gly Thr Cys Val Ser Ile Gly Tyr Gly Tyr Ala Asp Arg Ala Ser
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 Cys Lys Pro Lys Ser Ser Leu Glu Glu Thr Glu Val Leu Phe Val Phe
 1285 1290 1295
 Ile Gly Tyr Asp Arg Lys Ala Arg Thr His Asn Pro Tyr Lys Leu Ser
 1300 1305 1310
 Ser Thr Leu Thr Asn Ile Tyr Thr Gly Ser Arg Leu His Glu Ala Gly
 1315 1320 1325
 Cys Ala Pro Ser Tyr His Val Val Arg Gly Asp Ile Ala Thr Ala Thr
 1330 1335 1340
 Glu Gly Val Ile Ile Asn Ala Ala Asn Ser Lys Gly Gln Pro Gly Gly
 1345 1350 1355 1360
 Gly Val Cys Gly Ala Leu Tyr Lys Lys Phe Pro Glu Ser Phe Asp Leu
 1365 1370 1375
 Gln Pro Ile Glu Val Gly Lys Ala Arg Leu Val Lys Gly Ala Ala Lys
 1380 1385 1390
 His Ile Ile His Ala Val Gly Pro Asn Phe Asn Lys Val Ser Glu Val
 1395 1400 1405
 Glu Gly Asp Lys Gln Leu Ala Glu Ala Tyr Glu Ser Ile Ala Lys Ile
 1410 1415 1420
 Val Asn Asp Asn Asn Tyr Lys Ser Val Ala Ile Pro Leu Leu Ser Thr
 1425 1430 1435 1440
 Gly Ile Phe Ser Gly Asn Lys Asp Arg Leu Thr Gln Ser Leu Asn His
 1445 1450 1455
 Leu Leu Thr Ala Leu Asp Thr Thr Asp Ala Asp Val Ala Ile Tyr Cys
 1460 1465 1470
 Arg Asp Lys Lys Trp Glu Met Thr Leu Lys Glu Ala Val Ala Arg Arg
 1475 1480 1485
 Glu Ala Val Glu Glu Ile Cys Ile Ser Asp Asp Ser Ser Val Thr Glu
 1490 1495 1500
 Pro Asp Ala Glu Leu Val Arg Val His Pro Lys Ser Ser Leu Ala Gly
 1505 1510 1515 1520
 Arg Lys Gly Tyr Ser Thr Ser Asp Gly Lys Thr Phe Ser Tyr Leu Glu
 1525 1530 1535
 Gly Thr Lys Phe His Gln Ala Ala Lys Asp Ile Ala Glu Ile Asn Ala
 1540 1545 1550
 Met Trp Pro Val Ala Thr Glu Ala Asn Glu Gln Val Cys Met Tyr Ile
 1555 1560 1565
 Leu Gly Glu Ser Met Ser Ser Ile Arg Ser Lys Cys Pro Val Glu Glu
 1570 1575 1580
 Ser Glu Ala Ser Thr Pro Pro Ser Thr Leu Pro Cys Leu Cys Ile His
 1585 1590 1595 1600
 Ala Met Thr Pro Glu Arg Val Gln Arg Leu Lys Ala Ser Arg Pro Glu
 1605 1610 1615
 Gln Ile Thr Val Cys Ser Ser Phe Pro Leu Pro Lys Tyr Arg Ile Thr
 1620 1625 1630
 Gly Val Gln Lys Ile Gln Cys Ser Gln Pro Ile Leu Phe Ser Pro Lys
 1635 1640 1645
 Val Pro Ala Tyr Ile His Pro Arg Lys Tyr Leu Val Glu Thr Pro Pro
 1650 1655 1660
 Val Asp Glu Thr Pro Glu Pro Ser Ala Glu Asn Gln Ser Thr Glu Gly
 1665 1670 1675 1680
 Thr Pro Glu Gln Pro Pro Leu Ile Thr Glu Asp Glu Thr Arg Thr Arg
 1685 1690 1695

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 1700 1705 1710
 Leu Leu Ser Asp Gly Pro Thr His Gln Val Leu Gln Val Glu Ala Asp
 1715 1720 1725
 Ile His Gly Pro Pro Ser Val Ser Ser Ser Trp Ser Ile Pro His
 1730 1735 1740
 Ala Ser Asp Phe Asp Val Asp Ser Leu Ser Ile Leu Asp Thr Leu Glu
 1745 1750 1755 1760
 Gly Ala Ser Val Thr Ser Gly Ala Thr Ser Ala Glu Thr Asn Ser Tyr
 1765 1770 1775
 Phe Ala Lys Ser Met Glu Phe Leu Ala Arg Pro Val Pro Ala Pro Arg
 1780 1785 1790
 Thr Val Phe Arg Asn Pro Pro His Pro Ala Pro Arg Thr Arg Thr Pro
 1795 1800 1805
 Ser Leu Ala Pro Ser Arg Ala Cys Ser Arg Thr Ser Leu Val Ser Thr
 1810 1815 1820
 Pro Pro Gly Val Asn Arg Val Ile Thr Arg Glu Glu Leu Glu Ala Leu
 1825 1830 1835 1840
 Thr Pro Ser Arg Thr Pro Ser Arg Ser Val Ser Arg Thr Ser Leu Val
 1845 1850 1855
 Ser Asn Pro Pro Gly Val Asn Arg Val Ile Thr Arg Glu Glu Phe Glu
 1860 1865 1870
 Ala Phe Val Ala Gln Gln Arg Phe Asp Ala Gly Ala Tyr Ile Phe
 1875 1880 1885
 Ser Ser Asp Thr Gly Gln Gly His Leu Gln Gln Lys Ser Val Arg Gln
 1890 1895 1900
 Thr Val Leu Ser Glu Val Val Leu Glu Arg Thr Glu Leu Glu Ile Ser
 1905 1910 1915 1920
 Tyr Ala Pro Arg Leu Asp Gln Glu Lys Glu Glu Leu Leu Arg Lys Lys
 1925 1930 1935
 Leu Gln Leu Asn Pro Thr Pro Ala Asn Arg Ser Arg Tyr Gln Ser Arg
 1940 1945 1950
 Lys Val Glu Asn Met Lys Ala Ile Thr Ala Arg Arg Ile Leu Gln Gly
 1955 1960 1965
 Leu Gly His Tyr Leu Lys Ala Glu Gly Lys Val Glu Cys Tyr Arg Thr
 1970 1975 1980
 Leu His Pro Val Pro Leu Tyr Ser Ser Val Asn Arg Ala Phe Ser
 1985 1990 1995 2000
 Ser Pro Lys Val Ala Val Glu Ala Cys Asn Ala Met Leu Lys Glu Asn
 2005 2010 2015
 Phe Pro Thr Val Ala Ser Tyr Cys Ile Ile Pro Glu Tyr Asp Ala Tyr
 2020 2025 2030
 Leu Asp Met Val Asp Gly Ala Ser Cys Cys Leu Asp Thr Ala Ser Phe
 2035 2040 2045
 Cys Pro Ala Lys Leu Arg Ser Phe Pro Lys Lys His Ser Tyr Leu Glu
 2050 2055 2060
 Pro Thr Ile Arg Ser Ala Val Pro Ser Ala Ile Gln Asn Thr Leu Gln
 2065 2070 2075 2080
 Asn Val Leu Ala Ala Ala Thr Lys Arg Asn Cys Asn Val Thr Gln Met
 2085 2090 2095
 Arg Glu Leu Pro Val Leu Asp Ser Ala Ala Phe Asn Val Glu Cys Phe
 2100 2105 2110
 Lys Lys Tyr Ala Cys Asn Asn Glu Tyr Trp Glu Thr Phe Lys Glu Asn
 2115 2120 2125

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 Lys Gly Pro Lys Ala Ala Ala Leu Phe Ala Lys Thr His Asn Leu Asn
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 Met Leu Gln Asp Ile Pro Met Asp Arg Phe Val Met Asp Leu Lys Arg
 2165 2170 2175
 Asp Val Lys Val Thr Pro Gly Thr Lys His Thr Glu Glu Arg Pro Lys.
 2180 2185 2190
 Val Gln Val Ile Gln Ala Ala Asp Pro Leu Ala Thr Ala Tyr Leu Cys
 2195 2200 2205
 Gly Ile His Arg Glu Leu Val Arg Arg Leu Asn Ala Val Leu Leu Pro
 2210 2215 2220
 Asn Ile His Thr Leu Phe Asp Met Ser Ala Glu Asp Phe Asp Ala Ile
 2225 2230 2235 2240
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 Ala Ser Phe Asp Lys Ser Glu Asp Asp Ala Met Ala Leu Thr Ala Leu
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 Met Ile Leu Glu Asp Leu Gly Val Asp Ala Glu Leu Leu Thr Leu Ile
 2275 2280 2285
 Glu Ala Ala Phe Gly Glu Ile Ser Ser Ile His Leu Pro Thr Lys Thr
 2290 2295 2300
 Lys Phe Lys Phe Gly Ala Met Met Lys Ser Gly Met Phe Leu Thr Leu
 2305 2310 2315 2320
 Phe Val Asn Thr Val Ile Asn Ile Val Ile Ala Ser Arg Val Leu Arg
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 Glu Arg Leu Thr Gly Ser Pro Cys Ala Ala Phe Ile Gly Asp Asp Asn
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 2355 2360 2365
 Thr Trp Leu Asn Met Glu Val Lys Ile Ile Asp Ala Val Val Gly Glu
 2370 2375 2380
 Lys Ala Pro Tyr Phe Cys Gly Gly Phe Ile Leu Cys Asp Ser Val Thr
 2385 2390 2395 2400
 Gly Thr Ala Cys Arg Val Ala Asp Pro Leu Lys Arg Leu Phe Lys Leu
 2405 2410 2415
 Gly Lys Pro Leu Ala Ala Asp Asp Glu His Asp Asp Asp Arg Arg Arg
 2420 2425 2430
 Ala Leu His Glu Glu Ser Thr Arg Trp Asn Arg Val Gly Ile Leu Ser
 2435 2440 2445
 Glu Leu Cys Lys Ala Val Glu Ser Arg Tyr Glu Thr Val Gly Thr Ser
 2450 2455 2460
 Ile Ile Val Met Ala Met Thr Thr Leu Ala Ser Ser Val Lys Ser Phe
 2465 2470 2475 2480
 Ser Tyr Leu Arg Gly Ala Pro Ile Thr Leu Tyr Gly
 2485 2490

<210> 4

<211> 1476

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence; Note =
synthetic construct

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<221> CDS

<222> (1) ... (1476)

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 Met Ala Ala Arg Ala Ser Ile Leu Arg Gly Glu Lys Leu Asp Lys Trp
 1 5 10 15

gaa aag att agg tta agg cca ggg gga aag aaa cat tat atg tta aaa 96
 Glu Lys Ile Arg Leu Arg Pro Gly Gly Lys Lys His Tyr Met Leu Lys
 20 25 30

cac ata gta tgg gcg agc agg gag ctg gaa aga ttt gca ctt aac cct 144
 His Ile Val Trp Ala Ser Arg Glu Leu Glu Arg Phe Ala Leu Asn Pro
 35 40 45

ggc ctt tta gaa aca tca gaa gga tgt aaa caa ata atg aaa cag cta 192
 Gly Leu Leu Glu Thr Ser Glu Gly Cys Lys Gln Ile Met Lys Gln Leu
 50 55 60

caa cca gct ctc cag aca gga aca gag gaa ctt aaa tca tta tac aac 240
 Gln Pro Ala Leu Gln Thr Gly Thr Glu Glu Leu Lys Ser Leu Tyr Asn
 65 70 75 80

aca gta gca act ctc tat tgt gta cat gaa aag ata gaa gta cga gac 288
 Thr Val Ala Thr Leu Tyr Cys Val His Glu Lys Ile Glu Val Arg Asp
 85 90 95

acc aag gaa gcc tta gat aag ata gag gaa gaa caa aac aaa tgt cag 336
 Thr Lys Glu Ala Leu Asp Lys Ile Glu Glu Glu Gln Asn Lys Cys Gln
 100 105 110

caa aaa acg cag cag gca aaa gcg gct gac ggg aaa gtc agt caa aat 384
 Gln Lys Thr Gln Gln Ala Lys Ala Asp Gly Lys Val Ser Gln Asn
 115 120 125

tat cct ata gtg cag aat ctc caa ggg caa atg gta cat caa gcc ata 432
 Tyr Pro Ile Val Gln Asn Leu Gln Gly Gln Met Val His Gln Ala Ile
 130 135 140

tca cct aga acc ttg aat gca tgg gta aaa gta ata gaa gaa aag gct 480
 Ser Pro Arg Thr Leu Asn Ala Trp Val Lys Val Ile Glu Glu Lys Ala
 145 150 155 160

ttt agc cca gag gta ata ccc atg ttt aca gca tta tca gaa gga gcc 528
 Phe Ser Pro Glu Val Ile Pro Met Phe Thr Ala Leu Ser Glu Gly Ala
 165 170 175

acc cca caa gat tta aac acc atg tta aat aca gtg ggg gga cac caa 576
 Thr Pro Gln Asp Leu Asn Thr Met Leu Asn Thr Val Gly Gly His Gln
 180 185 190

gca gcc atg caa atg tta aaa gat act att aat gaa gag gct gca gaa 624
 Ala Ala Met Gln Met Leu Lys Asp Thr Ile Asn Glu Glu Ala Ala Glu
 195 200 205

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tgg gat aga tta cat cca gtc cat gcg ggg cct att gca cca ggc cag	210	215	220	672
Trp Asp Arg Leu His Pro Val His Ala Gly Pro Ile Ala Pro Gly Gln				
atg aga gaa cca agg gga agt gac ata gca gga act act agt acc ctt	225	230	235	720
Met Arg Glu Pro Arg Gly Ser Asp Ile Ala Gly Thr Thr Ser Thr Leu				
cag gaa caa ata gca tgg atg aca agt aac cca cct att cca gtg gga	245	250	255	768
Gln Glu Gln Ile Ala Trp Met Thr Ser Asn Pro Pro Ile Pro Val Gly				
gac atc tat aaa aga tgg ata att ctg ggg tta aat aaa ata gtg aga	260	265	270	816
Asp Ile Tyr Lys Arg Trp Ile Ile Leu Gly Leu Asn Lys Ile Val Arg				
atg tat agc ccg gtc agc att ttg gac ata aga caa ggg cca aag gaa	275	280	285	864
Met Tyr Ser Pro Val Ser Ile Leu Asp Ile Arg Gln Gly Pro Lys Glu				
ccc ttt cga gac tat gta gat cgg ttc ttt aaa act tta aga gct gaa	290	295	300	912
Pro Phe Arg Asp Tyr Val Asp Arg Phe Phe Lys Thr Leu Arg Ala Glu				
caa gct aca caa gaa gta aaa aat tgg atg aca gac acc ttg tta gtc	305	310	315	960
Gln Ala Thr Gln Glu Val Lys Asn Trp Met Thr Asp Thr Leu Leu Val				
caa aat gcg aac cca gat tgt aag acc att ttg aga gca tta gga cca	325	330	335	1008
Gln Asn Ala Asn Pro Asp Cys Lys Thr Ile Leu Arg Ala Leu Gly Pro				
ggg gct aca tta gaa gaa atg atg aca gca tgt caa ggg gtg gga gga	340	345	350	1056
Gly Ala Thr Leu Glu Met Met Thr Ala Cys Gln Gly Val Gly Gly				
cct ggc cac aaa gca aga gta ttg gct gag gca atg agt caa aca aac	355	360	365	1104
Pro Gly His Lys Ala Arg Val Leu Ala Glu Ala Met Ser Gln Thr Asn				
agt gga aac ata atg atg cag aga agc aat ttt aaa ggc cct aga aga	370	375	380	1152
Ser Gly Asn Ile Met Met Gln Arg Ser Asn Phe Lys Gly Pro Arg Arg				
att gtt aaa tgt ttt aac tgt ggc aag gaa ggg cac ata gcc aga aat	385	390	395	1200
Ile Val Lys Cys Phe Asn Cys Gly Lys Glu Gly His Ile Ala Arg Asn				
tgc aga gcc cct agg aaa aaa ggc tgt tgg aaa tgt gga aaa gaa gga	405	410	415	1248
Cys Arg Ala Pro Arg Lys Lys Gly Cys Trp Lys Cys Gly Lys Glu Gly				
cac caa atg aaa gac tgc act gag agg cag gct aat ttt tta ggg aaa	420	425	430	1296
His Gln Met Lys Asp Cys Thr Glu Arg Gln Ala Asn Phe Leu Gly Lys				

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att tgg cct tcc cac aag ggg agg cca ggg aat ttc ctt cag aac aga	1344		
Ile Trp Pro Ser His Lys Gly Arg Pro Gly Asn Phe Leu Gln Asn Arg			
435	440	445	
cca gag cca aca gcc cca cca gca gag agc ttc agg ttc gaa gag aca	1392		
Pro Glu Pro Thr Ala Pro Pro Ala Glu Ser Phe Arg Phe Glu Glu Thr			
450	455	460	
acc ccc gct ccg aaa cag gag ccg ata gaa agg gaa ccc tta act tcc	1440		
Thr Pro Ala Pro Lys Gln Glu Pro Ile Glu Arg Glu Pro Leu Thr Ser			
465	470	475	480
ctc aaa tca ctc ttt ggc agc gac ccc ttg tct caa	1476		
Leu Lys Ser Leu Phe Gly Ser Asp Pro Leu Ser Gln			
485	490		

<210> 5

<211> 492

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence; Note =
synthetic construct

<400> 5

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Glu Lys Ile Arg Leu Arg Pro Gly Gly Lys Lys His Tyr Met Leu Lys			
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His Ile Val Trp Ala Ser Arg Glu Leu Glu Arg Phe Ala Leu Asn Pro			
35	40	45	
Gly Leu Leu Glu Thr Ser Glu Gly Cys Lys Gln Ile Met Lys Gln Leu			
50	55	60	
Gln Pro Ala Leu Gln Thr Gly Thr Glu Glu Leu Lys Ser Leu Tyr Asn			
65	70	75	80
Thr Val Ala Thr Leu Tyr Cys Val His Glu Lys Ile Glu Val Arg Asp			
85	90	95	
Thr Lys Glu Ala Leu Asp Lys Ile Glu Glu Glu Gln Asn Lys Cys Gln			
100	105	110	
Gln Lys Thr Gln Gln Ala Lys Ala Ala Asp Gly Lys Val Ser Gln Asn			
115	120	125	
Tyr Pro Ile Val Gln Asn Leu Gln Gly Gln Met Val His Gln Ala Ile			
130	135	140	
Ser Pro Arg Thr Leu Asn Ala Trp Val Lys Val Ile Glu Glu Lys Ala			
145	150	155	160
Phe Ser Pro Glu Val Ile Pro Met Phe Thr Ala Leu Ser Glu Gly Ala			
165	170	175	
Thr Pro Gln Asp Leu Asn Thr Met Leu Asn Thr Val Gly Gly His Gln			
180	185	190	
Ala Ala Met Gln Met Leu Lys Asp Thr Ile Asn Glu Glu Ala Ala Glu			
195	200	205	

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Trp Asp Arg Leu His Pro Val His Ala Gly Pro Ile Ala Pro Gly Gln
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 Met Arg Glu Pro Arg Gly Ser Asp Ile Ala Gly Thr Thr Ser Thr Leu
 225 230 235 240
 Gln Glu Gln Ile Ala Trp Met Thr Ser Asn Pro Pro Ile Pro Val Gly
 245 250 255
 Asp Ile Tyr Lys Arg Trp Ile Ile Leu Gly Leu Asn Lys Ile Val Arg
 260 265 270
 Met Tyr Ser Pro Val Ser Ile Leu Asp Ile Arg Gln Gly Pro Lys Glu
 275 280 285
 Pro Phe Arg Asp Tyr Val Asp Arg Phe Phe Lys Thr Leu Arg Ala Glu
 290 295 300
 Gln Ala Thr Gln Glu Val Lys Asn Trp Met Thr Asp Thr Leu Leu Val
 305 310 315 320
 Gln Asn Ala Asn Pro Asp Cys Lys Thr Ile Leu Arg Ala Leu Gly Pro
 325 330 335
 Gly Ala Thr Leu Glu Glu Met Met Thr Ala Cys Gln Gly Val Gly Gly
 340 345 350
 Pro Gly His Lys Ala Arg Val Leu Ala Glu Ala Met Ser Gln Thr Asn
 355 360 365
 Ser Gly Asn Ile Met Met Gln Arg Ser Asn Phe Lys Gly Pro Arg Arg
 370 375 380
 Ile Val Lys Cys Phe Asn Cys Gly Lys Glu Gly His Ile Ala Arg Asn
 385 390 395 400
 Cys Arg Ala Pro Arg Lys Lys Gly Cys Trp Lys Cys Gly Lys Glu Gly
 405 410 415
 His Gln Met Lys Asp Cys Thr Glu Arg Gln Ala Asn Phe Leu Gly Lys
 420 425 430
 Ile Trp Pro Ser His Lys Gly Arg Pro Gly Asn Phe Leu Gln Asn Arg
 435 440 445
 Pro Glu Pro Thr Ala Pro Pro Ala Glu Ser Phe Arg Phe Glu Glu Thr
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 Thr Pro Ala Pro Lys Gln Glu Pro Ile Glu Arg Glu Pro Leu Thr Ser
 465 470 475 480
 Leu Lys Ser Leu Phe Gly Ser Asp Pro Leu Ser Gln
 485 490

<210> 6

<211> 813

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence; Note =
synthetic construct

<221> CDS

<222> (1)...(813)

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tcc aac atg gat gct gat tta tat ggg tat aaa tgg gct cgc gat aat	96
Ser Asn Met Asp Ala Asp Leu Tyr Gly Tyr Lys Trp Ala Arg Asp Asn	
20 25 30	
gtc ggg caa tca ggt gcg aca atc tat cga ttg tat ggg aag ccc gat	144
Val Gly Gln Ser Gly Ala Thr Ile Tyr Arg Leu Tyr Gly Lys Pro Asp	
35 40 45	
gct cca gag ttg ttt ctg aaa cat ggc aaa ggt agc gtt gcc aat gat	192
Ala Pro Glu Leu Phe Leu Lys His Gly Lys Gly Ser Val Ala Asn Asp	
50 55 60	
gtt aca gat gag atg gtc aga cta aac tgg ctg acg gaa ttt atg cct	240
Val Thr Asp Glu Met Val Arg Leu Asn Trp Leu Thr Glu Phe Met Pro	
65 70 75 80	
ctt ccg acc atc aag cat ttt atc cgt act cct gat gat gca tgg tta	288
Leu Pro Thr Ile Lys His Phe Ile Arg Thr Pro Asp Asp Ala Trp Leu	
85 90 95	
ctc acc act gct atc ccc ggg aaa aca gca ttc cag gta tta gaa gaa	336
Leu Thr Thr Ala Ile Pro Gly Lys Thr Ala Phe Gln Val Leu Glu Glu	
100 105 110	
tat cct gat tca ggt gaa aat att gtt gat gct ctc gta gtc ttc ctg	384
Tyr Pro Asp Ser Gly Glu Asn Ile Val Asp Ala Leu Ala Val Phe Leu	
115 120 125	
cgc cgg ttg cat tcg att cct gtt tgt aat tgt cct ttt aac agc gat	432
Arg Arg Leu His Ser Ile Pro Val Cys Asn Cys Pro Phe Asn Ser Asp	
130 135 140	
cgc gta ttt cgt ctc gct cag gct caa tca cga atg aat aac ggt ttg	480
Arg Val Phe Arg Leu Ala Gln Ala Gln Ser Arg Met Asn Asn Gly Leu	
145 150 155 160	
gtt gat gct agt gat ttt gat gac gag cgt aat ggc tgg cct gtt gaa	528
Val Asp Ala Ser Asp Phe Asp Asp Glu Arg Asn Gly Trp Pro Val Glu	
165 170 175	
caa gtc tgg aaa gaa atg cat aag ctt ttg cca ttc tca ccg gat tca	576
Gln Val Trp Lys Glu Met His Lys Leu Leu Pro Phe Ser Pro Asp Ser	
180 185 190	
gtc gtc act cat ggt gat ttc tca ctt gat aac ctt att ttt gac gag	624
Val Val Thr His Gly Asp Phe Ser Leu Asp Asn Leu Ile Phe Asp Glu	
195 200 205	
ggg aaa tta ata ggt tgt att gat gtt gga cga gtc gga atc gca gac	672
Gly Lys Leu Ile Gly Cys Ile Asp Val Gly Arg Val Gly Ile Ala Asp	
210 215 220	
cga tac cag gat ctt gcc atc cta tgg aac tgc ctc ggt gag ttt tct	720
Arg Tyr Gln Asp Leu Ala Ile Leu Trp Asn Cys Leu Gly Glu Phe Ser	
225 230 235 240	

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cct tca tta cag aaa cgg ctt ttt caa aaa tat ggt att gat aat cct 768
 Pro Ser Leu Gln Lys Arg Leu Phe Gln Lys Tyr Gly Ile Asp Asn Pro
 245 250 255

gat atg aat aaa ttg cag ttt cat ttg atg ctc gat gag ttt ttc 813
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 260 265 270

<210> 7

<211> 271

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence; Note =
 synthetic construct

<400> 7

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 Ser Asn Met Asp Ala Asp Leu Tyr Gly Tyr Lys Trp Ala Arg Asp Asn
 20 25 30
 Val Gly Gln Ser Gly Ala Thr Ile Tyr Arg Leu Tyr Gly Lys Pro Asp
 35 40 45
 Ala Pro Glu Leu Phe Leu Lys His Gly Lys Gly Ser Val Ala Asn Asp
 50 55 60
 Val Thr Asp Glu Met Val Arg Leu Asn Trp Leu Thr Glu Phe Met Pro
 65 70 75 80
 Leu Pro Thr Ile Lys His Phe Ile Arg Thr Pro Asp Asp Ala Trp Leu
 85 90 95
 Leu Thr Thr Ala Ile Pro Gly Lys Thr Ala Phe Gln Val Leu Glu Glu
 100 105 110
 Tyr Pro Asp Ser Gly Glu Asn Ile Val Asp Ala Leu Ala Val Phe Leu
 115 120 125
 Arg Arg Leu His Ser Ile Pro Val Cys Asn Cys Pro Phe Asn Ser Asp
 130 135 140
 Arg Val Phe Arg Leu Ala Gln Ala Ser Arg Met Asn Asn Gly Leu
 145 150 155 160
 Val Asp Ala Ser Asp Phe Asp Asp Glu Arg Asn Gly Trp Pro Val Glu
 165 170 175
 Gln Val Trp Lys Glu Met His Lys Leu Leu Pro Phe Ser Pro Asp Ser
 180 185 190
 Val Val Thr His Gly Asp Phe Ser Leu Asp Asn Leu Ile Phe Asp Glu
 195 200 205
 Gly Lys Leu Ile Gly Cys Ile Asp Val Gly Arg Val Gly Ile Ala Asp
 210 215 220
 Arg Tyr Gln Asp Leu Ala Ile Leu Trp Asn Cys Leu Gly Glu Phe Ser
 225 230 235 240
 Pro Ser Leu Gln Lys Arg Leu Phe Gln Lys Tyr Gly Ile Asp Asn Pro
 245 250 255
 Asp Met Asn Lys Leu Gln Phe His Leu Met Leu Asp Glu Phe Phe
 260 265 270

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<210> 8
 <211> 5076
 <212> DNA
 <213> Artificial Sequence

<220>

<223> Description of Artificial Sequence; Note =
 synthetic construct

<400> 8

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aggtagaagc	caagcagg	tc actgataatg	accatgt	taa tgccagagcg	ttttcgcatc	180
tggcttcaaa	actgatcgaa	acggaggtgg	acccatccga	cacgatc	ttt gacattggaa	240
gtgcggccgc	ccgcagaatg	tattctaagc	acaagtatca	ttgtatctgt	ccgatgagat	300
gtgcggaaga	tccggacaga	ttgtataagt	atgcaactaa	gctgaagaaaa	aactgttaagg	360
aaataactga	taaggaattg	gacaagaaaa	tgaaggagct	cgccgcccgtc	atgagcga	420
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aagtgcgtgt	ttaccaggat	gtatacgccg	ttgacggacc	ctataactct	ctacggctaa	540
cctgaatgg	ctacgacata	gtctagtc	ccaagatgtt	cccg	ttccag ccaatgtatc	600
cgatgcagcc	aatgccttat	cgcaacccgt	tcgccc	ccc g	gccc tggttcccc	660
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cgttcaagca	acgcgggac	gcgc	ccac	tttgc	gctaa	780
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agaaggctaa	gacagg	cc	tttcc	aa	aa gacca	900
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caatcatgtt	ggaagg	tttcc	tttcc	tttcc	tttcc	1020
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atcagg	gg	aaaaaaa	aaaaaaa	aaaaaaa	aaaaaaa	1980
cgctgategt	tttcc	aaaaaaa	aaaaaaa	aaaaaaa	aaaaaaa	2040
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gggcac	tttcc	aaaaaaa	aaaaaaa	aaaaaaa	aaaaaaa	2160
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ctgctgcaaa	tttcc	aaaaaaa	aaaaaaa	aaaaaaa	aaaaaaa	2460
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aggatgtgc	tttcc	aaaaaaa	aaaaaaa	aaaaaaa	aaaaaaa	2580
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acgttcc	tttcc	aaaaaaa	aaaaaaa	aaaaaaa	aaaaaaa	2700

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cagacaagcc cgtcaggcgcg cgtcagcggg tttggcggg tgcggggcg cagccatgac	3060
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<210> 9
 <211> 1026
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> Description of Artificial Sequence; Note =
 synthetic construct

<221> CDS
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Asn Pro Phe Ala Ala Pro Arg Arg Pro Trp Phe Pro Arg Thr Asp Pro	
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ttt ctg gcg atg cag gtg cag gaa tta acc cgc tcg atg gct aac ctg	144
Phe Leu Ala Met Gln Val Gln Glu Leu Thr Arg Ser Met Ala Asn Leu	
35 40 45	
acg ttc aag caa cgc cgg gac gcg cca cct gag ggg cca tcc gct aag	192
Thr Phe Lys Gln Arg Arg Asp Ala Pro Pro Glu Gly Pro Ser Ala Lys	
50 55 60	
aaa ccg aag aag gag gcc tcg caa aaa cag aaa ggg gga ggc caa ggg	240
Lys Pro Lys Lys Glu Ala Ser Gln Lys Gln Lys Gly Gly Gly Gln Gly	
65 70 75 80	
aag aag aag aac caa ggg aag aag aag gct aag aca ggg ccg cct	288
Lys Lys Lys Asn Gln Gly Lys Lys Lys Ala Lys Thr Gly Pro Pro	
85 90 95	
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Asn Pro Lys Ala Gln Asn Gly Asn Lys Lys Lys Thr Asn Lys Lys Pro	
100 105 110	
ggc aag aga cag cgc atg gtc atg aaa ttg gaa tct gac aag acg ttc	384
Gly Lys Arg Gln Arg Met Val Met Lys Leu Glu Ser Asp Lys Thr Phe	
115 120 125	
cca atc atg ttg gaa ggg aag ata aac ggc tac gct tgt gtg gtc gga	432
Pro Ile Met Leu Glu Gly Ile Asn Gly Tyr Ala Cys Val Val Gly	
130 135 140	
ggg aag tta ttc agg ccg atg cat gtg gaa ggc aag atc gac aac gac	480
Gly Lys Leu Phe Arg Pro Met His Val Glu Gly Lys Ile Asp Asn Asp	
145 150 155 160	
gtt ctg gcc gcg ctt aag acg aag aaa gca tcc aaa tac gat ctt gag	528
Val Leu Ala Ala Leu Lys Thr Lys Ala Ser Lys Tyr Asp Leu Glu	
165 170 175	
tat gca gat gtg cca cag aac atg cgg gcc gat aca ttc aaa tac acc	576
Tyr Ala Asp Val Pro Gln Asn Met Arg Ala Asp Thr Phe Lys Tyr Thr	
180 185 190	
cat gag aaa ccc caa ggc tat tac agc tgg cat cat gga gca gtc caa	624
His Glu Lys Pro Gln Gly Tyr Tyr Ser Trp His His Gly Ala Val Gln	
195 200 205	
tat gaa aat ggg cgt ttc acg gtg ccg aaa gga gtt ggg gcc aag gga	672
Tyr Glu Asn Gly Arg Phe Thr Val Pro Lys Gly Val Gly Ala Lys Gly	
210 215 220	

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gag	caa	tgg	tca	cta	gtg	acc	acc	atg	tgt	ctg	ctc	gcc	aat	gtg	acg	864
Glu	Gln	Trp	Ser	Leu	Val	Thr	Thr	Met	Cys	Leu	Leu	Ala	Asn	Val	Thr	
				275				280				285				
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Phe	Pro	Cys	Ala	Gln	Pro	Pro	Ile	Cys	Tyr	Asp	Arg	Lys	Pro	Ala	Glu	
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Thr	Leu	Ala	Met	Leu	Ser	Val	Asn	Ile	Pro	Ala	Gly	Arg	Ile	Ser	Arg	
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Asn	Tyr	Tyr	Asn	Trp	Leu	Gly	Ala	Gly	Tyr	Tyr	Cys	Gly	His	Val	Arg	
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Ala	Asp	Gln	Pro	Glu	Thr											
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<210> 10
 <211> 342
 <212> PRT
 <213> Artificial Sequence

<220>
 <223> Description of Artificial Sequence; Note =
 synthetic construct

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 35 40 45
 Thr Phe Lys Gln Arg Arg Asp Ala Pro Pro Glu Gly Pro Ser Ala Lys
 50 55 60
 Lys Pro Lys Lys Glu Ala Ser Gln Lys Gln Lys Gly Gly Gly Gln Gly
 65 70 75 80

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Lys Lys Lys Lys Asn Gln Gly Lys Lys Ala Lys Thr Gly Pro Pro
 85 90 95
 Asn Pro Lys Ala Gln Asn Gly Asn Lys Lys Thr Asn Lys Lys Pro
 100 105 110
 Gly Lys Arg Gln Arg Met Val Met Lys Leu Glu Ser Asp Lys Thr Phe
 115 120 125
 Pro Ile Met Leu Glu Gly Lys Ile Asn Gly Tyr Ala Cys Val Val Gly
 130 135 140
 Gly Lys Leu Phe Arg Pro Met His Val Glu Gly Lys Ile Asp Asn Asp
 145 150 155 160
 Val Leu Ala Ala Leu Lys Thr Lys Lys Ala Ser Lys Tyr Asp Leu Glu
 165 170 175
 Tyr Ala Asp Val Pro Gln Asn Met Arg Ala Asp Thr Phe Lys Tyr Thr
 180 185 190
 His Glu Lys Pro Gln Gly Tyr Tyr Ser Trp His His Gly Ala Val Gln
 195 200 205
 Tyr Glu Asn Gly Arg Phe Thr Val Pro Lys Gly Val Gly Ala Lys Gly
 210 215 220
 Asp Ser Gly Arg Pro Ile Leu Asp Asn Gln Gly Arg Val Val Ala Ile
 225 230 235 240
 Val Leu Gly Gly Val Asn Glu Gly Ser Arg Thr Ala Leu Ser Val Val
 245 250 255
 Met Trp Asn Glu Lys Gly Val Thr Val Lys Tyr Thr Pro Glu Asn Cys
 260 265 270
 Glu Gln Trp Ser Leu Val Thr Thr Met Cys Leu Leu Ala Asn Val Thr
 275 280 285
 Phe Pro Cys Ala Gln Pro Pro Ile Cys Tyr Asp Arg Lys Pro Ala Glu
 290 295 300
 Thr Leu Ala Met Leu Ser Val Asn Ile Pro Ala Gly Arg Ile Ser Arg
 305 310 315 320
 Asn Tyr Tyr Asn Trp Leu Gly Ala Gly Tyr Tyr Cys Gly His Val Arg
 325 330 335
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<210> 11

<211> 6989

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence; Note =
synthetic construct

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<210> 12
<211> 2943
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence; Note =
synthetic construct

<221> CDS
<222> (1) . . . (2943)

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 tgt gct caa cca cca att tgc tac gac aga aaa cca gca gag act ttg 96
 Cys Ala Gln Pro Pro Ile Cys Tyr Asp Arg Lys Pro Ala Glu Thr Leu
 20 25 30

 gcc atg ctc agc gtt aac gtt gac aac ccg ggc tac gat gag ctg ctg 144
 Ala Met Leu Ser Val Asn Val Asp Asn Pro Gly Tyr Asp Glu Leu Leu
 35 40 45

 gaa gca gct gtt aag tgc ccc gga agg aaa agg aga tcc acc gag gag 192
 Glu Ala Ala Val Lys Cys Pro Gly Arg Lys Arg Arg Ser Thr Glu Glu
 50 55 60

 ctg ttt aag gag tat aag cta acg cgc cct tac atg gcc aga tgc atc 240
 Leu Phe Lys Glu Tyr Lys Leu Thr Arg Pro Tyr Met Ala Arg Cys Ile
 65 70 75 80

 aga tgt gca gtt ggg agc tgc cat agt cca ata gca atc gag gca gta 288
 Arg Cys Ala Val Gly Ser Cys His Ser Pro Ile Ala Ile Glu Ala Val
 85 90 95

 aag agc gac ggg cac gac ggt tat gtt aga ctt cag act tcc tcg cag 336
 Lys Ser Asp Gly His Asp Gly Tyr Val Arg Leu Gln Thr Ser Ser Gln
 100 105 110

 tat ggc ctg gat tcc tcc ggc aac tta aag ggc agg acc atg cgg tat 384
 Tyr Gly Leu Asp Ser Ser Gly Asn Leu Lys Gly Arg Thr Met Arg Tyr
 115 120 125

 gac atg cac ggg acc att aaa gag ata cca cta cat caa gtg tca ctc 432
 Asp Met His Gly Thr Ile Lys Glu Ile Pro Leu His Gln Val Ser Leu
 130 135 140

 cat aca tct cgc ccg tgt cac att gtg gat ggg cac ggt tat ttc ctg 480
 His Thr Ser Arg Pro Cys His Ile Val Asp Gly His Gly Tyr Phe Leu
 145 150 155 160
 ctt gcc agg tgc ccg gca ggg gac tcc atc acc atg gaa ttt aag aaa 528
 Leu Ala Arg Cys Pro Ala Gly Asp Ser Ile Thr Met Glu Phe Lys Lys
 165 170 175

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gat tcc gtc aca cac tcc tgc tcg gtg ccg tat gaa gtg aaa ttt aat	576
Asp Ser Val Thr His Ser Cys Ser Val Pro Tyr Glu Val Lys Phe Asn	
180	185
190	
cct gta ggc aga gaa ctc tat act cat ccc cca gaa cac gga gta gag	624
Pro Val Gly Arg Glu Leu Tyr Thr His Pro Pro Glu His Gly Val Glu	
195	200
205	
caa gcg tgc caa gtc tac gca cat gat gca cag aac aga gga gct tat	672
Gln Ala Cys Gln Val Tyr Ala His Asp Ala Gln Asn Arg Gly Ala Tyr	
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220	
gtc gag atg cac ctc cca ggc tca gaa gtg gac agc agt ttg gtt tcc	720
Val Glu Met His Leu Pro Gly Ser Glu Val Asp Ser Ser Leu Val Ser	
225	230
235	240
ttg agc ggc agt tca gtc acc gtg aca cct cct gtt ggg act agc gcc	768
Leu Ser Gly Ser Ser Val Thr Val Thr Pro Pro Val Gly Thr Ser Ala	
245	250
255	
ctg gtg gaa tgc gag tgt ggc ggc aca aag atc tcc aag acc atc aac	816
Leu Val Glu Cys Glu Cys Gly Gly Thr Lys Ile Ser Lys Thr Ile Asn	
260	265
270	
aag aca aaa cag ttc agc cag tgc aca aag aag gag cag tgc aga gca	864
Lys Thr Lys Gln Phe Ser Gln Cys Thr Lys Lys Glu Gln Cys Arg Ala	
275	280
285	
tat cgg ctg cag aac gat aag tgg gtg tat aat tct gac aaa ctg ccc	912
Tyr Arg Leu Gln Asn Asp Lys Trp Val Tyr Asn Ser Asp Lys Leu Pro	
290	295
300	
aaa gca gcg gga gcc acc tta aaa gga aaa ctg cat gtc cca ttc ttg	960
Lys Ala Ala Gly Ala Thr Leu Lys Gly Lys Leu His Val Pro Phe Leu	
305	310
315	320
ctg gca gac ggc aaa tgc acc gtg cct cta gca cca gaa cct atg ata	1008
Leu Ala Asp Gly Lys Cys Thr Val Pro Leu Ala Pro Glu Pro Met Ile	
325	330
335	
acc ttc ggt ttc aga tca gtg tca ctg aaa ctg cac ccc cct aag aat ccc	1056
Thr Phe Gly Phe Arg Ser Val Ser Leu Lys Leu His Pro Lys Asn Pro	
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350	
aca tat cta acc acc cgc caa ctt gct gat gag cct cac tac acg cat	1104
Thr Tyr Leu Thr Thr Arg Gln Leu Ala Asp Glu Pro His Tyr Thr His	
355	360
365	
gag ctc ata tct gaa cca gct gtt agg aat ttt acc gtc acc gga aaa	1152
Glu Leu Ile Ser Glu Pro Ala Val Arg Asn Phe Thr Val Thr Gly Lys	
370	375
380	
ggg tgg gag ttt gta tgg gga aac cac ccg ccg aaa agg ttt tgg gca	1200
Gly Trp Glu Phe Val Trp Gly Asn His Pro Pro Lys Arg Phe Trp Ala	
385	390
395	400

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act cat tat tac cac aga tac cct atg tcc acc atc ctg ggt ttg tca Thr His Tyr Tyr His Arg Tyr Pro Met Ser Thr Ile Leu Gly Leu Ser 420 425 430	1296
att tgt gcc gcc att gca acc gtt tcc gtt gca gcg tct acc tgg ctg Ile Cys Ala Ala Ile Ala Thr Val Ser Val Ala Ala Ser Thr Trp Leu 435 440 445	1344
ttt tgc aga tct aga gtt gcg tgc cta act cct tac cgg cta aca cct Phe Cys Arg Ser Arg Val Ala Cys. Leu Thr Pro Tyr Arg Leu Thr Pro 450 455 460	1392
aac gct agg ata cca ttt tgt ctg gct gtg ctt tgc tgc gcc cgcc act Asn Ala Arg Ile Pro Phe Cys Leu Ala Val Leu Cys Cys Ala Arg Thr 465 470 475 480	1440
gcc cggtt ggc gag acc acc tgg gag tcc ttg gat cac cta tgg aac aat Ala Arg Ala Glu Thr Trp Glu Ser Leu Asp His Leu Trp Asn Asn 485 490 495	1488
aac caa cag atg ttc tgg att caa ttg ctg atc cct ctg gcc gcc ttg Asn Gln Gln Met Phe Trp Ile Gln Leu Leu Ile Pro Leu Ala Ala Leu 500 505 510	1536
atc gta gtg act cgcc ctg ctc agg tgc gtg tgc tgt gtc gtg cct ttt Ile Val Val Thr Arg Leu Leu Arg Cys Val Cys Cys Val Val Pro Phe 515 520 525	1584
tta gtc atg gcc ggc gcc gca ggc ggc gcc tac gag cac ggc acc Leu Val Met Ala Gly Ala Ala Gly Ala Tyr Glu His Ala Thr 530 535 540	1632
acg atg ccg agc caa gcg gga atc tcg tat aac act ata gtc aac aga Thr Met Pro Ser Gln Ala Gly Ile Ser Tyr Asn Thr Ile Val Asn Arg 545 550 555 560	1680
gca ggc tac gca cca ctc cct atc agc ata aca cca aca aag atc aag Ala Gly Tyr Ala Pro Leu Pro Ile Ser Ile Thr Pro Thr Lys Ile Lys 565 570 575	1728
ctg ata cct aca gtg aac ttg gag tac gtc acc tgc cac tac aaa aca Leu Ile Pro Thr Val Asn Leu Glu Tyr Val Thr Cys His Tyr Lys Thr 580 585 590	1776
gga atg gat tca cca gcc atc aaa tgc tgc gga tct cag gaa tgc act Gly Met Asp Ser Pro Ala Ile Lys Cys Cys Gly Ser Gln Glu Cys Thr 595 600 605	1824
cca act tac agg cct gat gaa cag tgc aaa gtc ttc aca ggg gtt tac Pro Thr Tyr Arg Pro Asp Glu Gln Cys Lys Val Phe Thr Gly Val Tyr 610 615 620	1872

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ccg ttc atg tgg ggt gca tat tgc ttt tgc gac act gag aac acc Pro Phe Met Trp Gly Gly Ala Tyr Cys Phe Cys Asp Thr Glu Asn Thr 625 630 635 640	1920
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gga gaa act cct gtg aat ttc aat ggg gtc aaa tta act gca ggt ccg Gly Glu Thr Pro Val Asn Phe Asn Gly Val Lys Leu Thr Ala Gly Pro 690 695 700	2112
ctt tcc aca gct tgg aca ccc ttt gat cgc aaa atc gtg cag tat gcc Leu Ser Thr Ala Trp Thr Pro Phe Asp Arg Lys Ile Val Gln Tyr Ala 705 710 715 720	2160
ggg gag atc tat aat tat gat ttt cct gag tat ggg gca gga caa cca Gly Glu Ile Tyr Asn Tyr Asp Phe Pro Glu Tyr Gly Ala Gly Gln Pro 725 730 735	2208
gga gca ttt gga gat ata caa tcc aga aca gtc tca agc tca gat ctg Gly Ala Phe Gly Asp Ile Gln Ser Arg Thr Val Ser Ser Asp Leu 740 745 750	2256
tat gcc aat acc aac cta gtg ctg cag aga ccc aaa gca gga gcg atc Tyr Ala Asn Thr Asn Leu Val Leu Gln Arg Pro Lys Ala Gly Ala Ile 755 760 765	2304
cac gtg cca tac act cag gca cct tcg ggt ttt gag caa tgg aag aaa His Val Pro Tyr Thr Gln Ala Pro Ser Gly Phe Glu Gln Trp Lys Lys 770 775 780	2352
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tta gcc ttt gac att ccc gac gcc ttg ttc acc agg gtg tca gaa aca Leu Ala Phe Asp Ile Pro Asp Ala Leu Phe Thr Arg Val Ser Glu Thr 820 825 830	2496
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865	870	875	880	2640
gca gca gtc gag cta acc gag caa ggg tcg gcg act atc cat ttc tcg Ala Ala Val Glu Leu Thr Glu Gln Gly Ser Ala Thr Ile His Phe Ser	885	890	895	2688
900	905	910		2736
gtt acg tgc aaa ggt gat tgt cac ccc ccg aaa gac cat att gtg aca Val Thr Cys Lys Gly Asp Cys His Pro Pro Lys Asp His Ile Val Thr	915	920	925	2784
930	935	940		2832
945	950	955	960	2880
ata att ggc ttg gtg ctg gct act att gtg gcc atg tac gtg ctg acc Ile Ile Gly Leu Val Leu Ala Thr Ile Val Ala Met Tyr Val Leu Thr	965	970	975	2928
980				2943
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<211> 981
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence; Note =
synthetic construct

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 Ala Met Leu Ser Val Asn Val Asp Asn Pro Gly Tyr Asp Glu Leu Leu
 35 40 45

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 Arg Cys Ala Val Gly Ser Cys His Ser Pro Ile Ala Ile Glu Ala Val
 85 90 95
 Lys Ser Asp Gly His Asp Gly Tyr Val Arg Leu Gln Thr Ser Ser Gln
 100 105 110
 Tyr Gly Leu Asp Ser Ser Gly Asn Leu Lys Gly Arg Thr Met Arg Tyr
 115 120 125
 Asp Met His Gly Thr Ile Lys Glu Ile Pro Leu His Gln Val Ser Leu
 130 135 140
 His Thr Ser Arg Pro Cys His Ile Val Asp Gly His Gly Tyr Phe Leu
 145 150 155 160
 Leu Ala Arg Cys Pro Ala Gly Asp Ser Ile Thr Met Glu Phe Lys Lys
 165 170 175
 Asp Ser Val Thr His Ser Cys Ser Val Pro Tyr Glu Val Lys Phe Asn
 180 185 190
 Pro Val Gly Arg Glu Leu Tyr Thr His Pro Pro Glu His Gly Val Glu
 195 200 205
 Gln Ala Cys Gln Val Tyr Ala His Asp Ala Gln Asn Arg Gly Ala Tyr
 210 215 220
 Val Glu Met His Leu Pro Gly Ser Glu Val Asp Ser Ser Leu Val Ser
 225 230 235 240
 Leu Ser Gly Ser Ser Val Thr Val Thr Pro Pro Val Gly Thr Ser Ala
 245 250 255
 Leu Val Glu Cys Glu Cys Gly Gly Thr Lys Ile Ser Lys Thr Ile Asn
 260 265 270
 Lys Thr Lys Gln Phe Ser Gln Cys Thr Lys Lys Glu Gln Cys Arg Ala
 275 280 285
 Tyr Arg Leu Gln Asn Asp Lys Trp Val Tyr Asn Ser Asp Lys Leu Pro
 290 295 300
 Lys Ala Ala Gly Ala Thr Leu Lys Gly Lys Leu His Val Pro Phe Leu
 305 310 315 320
 Leu Ala Asp Gly Lys Cys Thr Val Pro Leu Ala Pro Glu Pro Met Ile
 325 330 335
 Thr Phe Gly Phe Arg Ser Val Ser Leu Lys Leu His Pro Lys Asn Pro
 340 345 350
 Thr Tyr Leu Thr Thr Arg Gln Leu Ala Asp Glu Pro His Tyr Thr His
 355 360 365
 Glu Leu Ile Ser Glu Pro Ala Val Arg Asn Phe Thr Val Thr Gly Lys
 370 375 380
 Gly Trp Glu Phe Val Trp Gly Asn His Pro Pro Lys Arg Phe Trp Ala
 385 390 395 400
 Gln Glu Thr Ala Pro Gly Asn Pro His Gly Leu Pro His Glu Val Ile
 405 410 415
 Thr His Tyr Tyr His Arg Tyr Pro Met Ser Thr Ile Leu Gly Leu Ser
 420 425 430
 Ile Cys Ala Ala Ile Ala Thr Val Ser Val Ala Ala Ser Thr Trp Leu
 435 440 445
 Phe Cys Arg Ser Arg Val Ala Cys Leu Thr Pro Tyr Arg Leu Thr Pro
 450 455 460
 Asn Ala Arg Ile Pro Phe Cys Leu Ala Val Leu Cys Cys Ala Arg Thr
 465 470 475 480

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Ala Arg Ala Glu Thr Thr Trp Glu Ser Leu Asp His Leu Trp Asn Asn
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 Asn Gln Gln Met Phe Trp Ile Gln Leu Leu Ile Pro Leu Ala Ala Leu
 500 505 510
 Ile Val Val Thr Arg Leu Leu Arg Cys Val Cys Cys Val Val Pro Phe
 515 520 525
 Leu Val Met Ala Gly Ala Ala Gly Ala Tyr Glu His Ala Thr
 530 535 540
 Thr Met Pro Ser Gln Ala Gly Ile Ser Tyr Asn Thr Ile Val Asn Arg
 545 550 555 560
 Ala Gly Tyr Ala Pro Leu Pro Ile Ser Ile Thr Pro Thr Lys Ile Lys
 565 570 575
 Leu Ile Pro Thr Val Asn Leu Glu Tyr Val Thr Cys His Tyr Lys Thr
 580 585 590
 Gly Met Asp Ser Pro Ala Ile Lys Cys Cys Gly Ser Gln Glu Cys Thr
 595 600 605
 Pro Thr Tyr Arg Pro Asp Glu Gln Cys Lys Val Phe Thr Gly Val Tyr
 610 615 620
 Pro Phe Met Trp Gly Gly Ala Tyr Cys Phe Cys Asp Thr Glu Asn Thr
 625 630 635 640
 Gln Val Ser Lys Ala Tyr Val Met Lys Ser Asp Asp Cys Leu Ala Asp
 645 650 655
 His Ala Glu Ala Tyr Lys Ala His Thr Ala Ser Val Gln Ala Phe Leu
 660 665 670
 Asn Ile Thr Val Gly Glu His Ser Ile Val Thr Thr Val Tyr Val Asn
 675 680 685
 Gly Glu Thr Pro Val Asn Phe Asn Gly Val Lys Leu Thr Ala Gly Pro
 690 695 700
 Leu Ser Thr Ala Trp Thr Pro Phe Asp Arg Lys Ile Val Gln Tyr Ala
 705 710 715 720
 Gly Glu Ile Tyr Asn Tyr Asp Phe Pro Glu Tyr Gly Ala Gly Gln Pro
 725 730 735
 Gly Ala Phe Gly Asp Ile Gln Ser Arg Thr Val Ser Ser Asp Leu
 740 745 750
 Tyr Ala Asn Thr Asn Leu Val Leu Gln Arg Pro Lys Ala Gly Ala Ile
 755 760 765
 His Val Pro Tyr Thr Gln Ala Pro Ser Gly Phe Glu Gln Trp Lys Lys
 770 775 780
 Asp Lys Ala Pro Ser Leu Lys Phe Thr Ala Pro Phe Gly Cys Glu Ile
 785 790 795 800
 Tyr Thr Asn Pro Ile Arg Ala Glu Asn Cys Thr Val Gly Ser Ile Pro
 805 810 815
 Leu Ala Phe Asp Ile Pro Asp Ala Leu Phe Thr Arg Val Ser Glu Thr
 820 825 830
 Pro Thr Leu Ser Ala Ala Glu Cys Thr Leu Asn Glu Cys Val Tyr Ser
 835 840 845
 Ser Asp Phe Gly Gly Ile Ala Thr Val Lys Tyr Ser Ala Ser Lys Ser
 850 855 860
 Gly Lys Cys Ala Val His Val Pro Ser Gly Thr Ala Thr Leu Lys Glu
 865 870 875 880
 Ala Ala Val Glu Leu Thr Glu Gln Gly Ser Ala Thr Ile His Phe Ser
 885 890 895
 Thr Ala Asn Ile His Pro Glu Phe Arg Leu Gln Ile Cys Thr Ser Tyr
 900 905 910

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Val Thr Cys Lys Gly Asp Cys His Pro Pro Lys Asp His Ile Val Thr
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 His Pro Gln Tyr His Ala Gln Thr Phe Thr Ala Ala Val Ser Lys Thr
 930 935 940
 Ala Trp Thr Trp Leu Thr Ser Leu Leu Gly Gly Ser Ala Val Ile Ile
 945 950 955 960
 Ile Ile Gly Leu Val Leu Ala Thr Ile Val Ala Met Tyr Val Leu Thr
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 Asn Gln Lys His Asn
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<210> 14

<211> 12379

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence; Note =
 synthetic construct

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 Leu Asn Lys Arg Thr Gln Asp Phe Trp Glu Val Gln Leu Gly Ile Pro
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 His Pro Ala Gly Leu Lys Lys Lys Ser Val Thr Val Leu Asp Val
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taagttttaa	tgcggttagtt	tatcacagtt	aaattctaa	cgcagtcagg	13500
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13584

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<211> 2532

<212> DNA

<213> Artificial Sequence

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synthetic construct

<221> CDS

<222> (1) ... (2532)

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1 5 10 15

48

ggc acc tta ggc ttt tgg atg ata ata att tgt agg gtg gtg ggg aac
Gly Thr Leu Gly Phe Trp Met Ile Ile Cys Arg Val Val Gly Asn
20 25 30

96

ttg aac ttg tgg gtc aca gtc tat tat ggg gta cct gtg tgg aaa gaa
Leu Asn Leu Trp Val Thr Val Tyr Tyr Gly Val Pro Val Trp Lys Glu
35 40 45

144

gca aaa act act cta ttc tgt gca tca gat gct aaa gca tat gat aaa
Ala Lys Thr Thr Leu Phe Cys Ala Ser Asp Ala Lys Ala Tyr Asp Lys
50 55 60

192

gaa gta cat aat gtc tgg gct aca cat gcc tgt gta ccc aca gac ccc
Glu Val His Asn Val Trp Ala Thr His Ala Cys Val Pro Thr Asp Pro
65 70 75 80

240

aac cca cga gaa ata gtt ttg gaa aat gta aca gaa aat ttt aac atg
Asn Pro Arg Glu Ile Val Leu Glu Asn Val Thr Glu Asn Phe Asn Met
85 90 95

288

tgg aaa aat gac atg gtg gat cag atg cat gag gat ata atc agt tta
Trp Lys Asn Asp Met Val Asp Gln Met His Glu Asp Ile Ile Ser Leu
100 105 110

336

tgg gat caa agc cta aaa cca tgt gta aag ttg acc cca ctc tgt gtc
Trp Asp Gln Ser Leu Lys Pro Cys Val Lys Leu Thr Pro Leu Cys Val
115 120 125

384

act tta aat tgt aca aat gca cct gcc tac aat aat agc atg cat gga
Thr Leu Asn Cys Thr Asn Ala Pro Ala Tyr Asn Asn Ser Met His Gly
130 135 140

432

gaa atg aaa aat tgc tct ttc aat aca acc aca gag ata aga gat agg
Glu Met Lys Asn Cys Ser Phe Asn Thr Thr Glu Ile Arg Asp Arg
145 150 155 160

480

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aaa cag aaa gcg tat gca ctt ttt tat aaa cct gat gta gtg cca ctt	528
Lys Gln Lys Ala Tyr Ala Leu Phe Tyr Lys Pro Asp Val Val Pro Leu	
165 170 175	
aat agg aga gaa gag aat aat ggg aca gga gag tat ata tta ata aat	576
Asn Arg Arg Glu Glu Asn Asn Gly Thr Gly Glu Tyr Ile Leu Ile Asn	
180 185 190	
tgc aat tcc tca acc ata aca caa gcc tgt cca aag gtc act ttt gac	624
Cys Asn Ser Ser Thr Ile Thr Gln Ala Cys Pro Lys Val Thr Phe Asp	
195 200 205	
cca att cct ata cat tat tgt gct cca gct ggt tat gcg att cta aag	672
Pro Ile Pro Ile His Tyr Cys Ala Pro Ala Gly Tyr Ala Ile Leu Lys	
210 215 220	
tgt aat aat aag aca ttc aat ggg aca gga cca tgc aat aat gtc agc	720
Cys Asn Asn Lys Thr Phe Asn Gly Thr Gly Pro Cys Asn Asn Val Ser	
225 230 235 240	
aca gta caa tgt aca cat gga att atg cca gtg gta tca act caa tta	768
Thr Val Gln Cys Thr His Gly Ile Met Pro Val Val Ser Thr Gln Leu	
245 250 255	
ctg tta aat ggt agc cta gca gaa gaa gag ata ata att aga tct gaa	816
Leu Leu Asn Gly Ser Leu Ala Glu Glu Glu Ile Ile Ile Arg Ser Glu	
260 265 270	
aat ctg aca aac aat atc aaa aca ata ata gtc cac ctt aat aaa tct	864
Asn Leu Thr Asn Asn Ile Lys Thr Ile Ile Val His Leu Asn Lys Ser	
275 280 285	
gta gaa att gtg tgt aca aga ccc aac aat aat aca aga aaa agt ata	912
Val Glu Ile Val Cys Thr Arg Pro Asn Asn Asn Thr Arg Lys Ser Ile	
290 295 300	
agg ata gga cca gga caa aca ttc tat gca aca ggt gaa ata ata gga	960
Arg Ile Gly Pro Gly Gln Thr Phe Tyr Ala Thr Gly Glu Ile Ile Gly	
305 310 315 320	
aac ata aga gaa gca cat tgt aac att agt aaa agt aac tgg acc agt	1008
Asn Ile Arg Glu Ala His Cys Asn Ile Ser Lys Ser Asn Trp Thr Ser	
325 330 335	
act tta gaa cag gta aag aaa aaa tta aaa gaa cac tac aat aag aca	1056
Thr Leu Glu Gln Val Lys Lys Lys Leu Lys Glu His Tyr Asn Lys Thr	
340 345 350	
ata gaa ttt aac cca ccc tca gga ggg gat cta gaa gtt aca aca cat	1104
Ile Glu Phe Asn Pro Pro Ser Gly Gly Asp Leu Glu Val Thr Thr His	
355 360 365	
agc ttt aat tgt aga gga gaa ttt ttc tat tgc aat aca aca aaa ctg	1152
Ser Phe Asn Cys Arg Gly Glu Phe Phe Tyr Cys Asn Thr Thr Lys Leu	
370 375 380	

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aag ata aaa caa att ata aac atg tgg cag aag gta gga cga gca atg Lys Ile Lys Gln Ile Ile Asn Met Trp Gln Lys Val Gly Arg Ala Met 405 410 415	1248
tat gcc cct ccc att gaa gga aac ata aca tgt aaa tca aat atc aca Tyr Ala Pro Pro Ile Glu Gly Asn Ile Thr Cys Lys Ser Asn Ile Thr 420 425 430	1296
gga cta cta ttg aca cgt gat gga gga aag aat aca aca aat gag ata Gly Leu Leu Leu Thr Arg Asp Gly Gly Lys Asn Thr Thr Asn Glu Ile 435 440 445	1344
ttc aga ccg gga gga aat atg aag gac aat tgg aga agt gaa tta Phe Arg Pro Gly Gly Asn Met Lys Asp Asn Trp Arg Ser Glu Leu 450 455 460	1392
tat aaa tat aaa gtg gta gaa att gag cca ttg gga gta gca ccc act Tyr Lys Tyr Lys Val Val Glu Ile Glu Pro Leu Gly Val Ala Pro Thr 465 470 475 480	1440
aaa tca aaa agg aga gtg gtg gag aga gaa aaa aga gca gtg gga cta Lys Ser Lys Arg Arg Val Val Glu Arg Glu Lys Arg Ala Val Gly Leu 485 490 495	1488
gga gct gta ctc ctt ggg ttc ttg gga gca gca gga agc act atg ggc Gly Ala Val Leu Leu Gly Phe Leu Gly Ala Ala Gly Ser Thr Met Gly 500 505 510	1536
gcg gcg tca ata acg ctg acg gta cag gcc aga caa ctg ttg tct ggt Ala Ala Ser Ile Thr Leu Thr Val Gln Ala Arg Gln Leu Leu Ser Gly 515 520 525	1584
ata gtg caa cag caa agc aat ttg ctg aga gct ata gag gcg caa cag Ile Val Gln Gln Ser Asn Leu Ile Arg Ala Ile Glu Ala Gln Gln 530 535 540	1632
cat atg ttg caa ctc acg gtc tgg ggc att aag cag ctc cag aca aga His Met Leu Gln Leu Thr Val Trp Gly Ile Lys Gln Leu Gln Thr Arg 545 550 555 560	1680
gtc ttg gct ata gag aga tac cta aag gat caa cag ctc cta ggg ctt Val Leu Ala Ile Glu Arg Tyr Leu Lys Asp Gln Gln Leu Leu Gly Leu 565 570 575	1728
tgg ggc tgc tct gga aaa atc atc tgc acc act gct gtg cct tgg aac Trp Gly Cys Ser Gly Lys Ile Ile Cys Thr Thr Ala Val Pro Trp Asn 580 585 590	1776
tcc agt tgg agt aat aaa tct caa gaa gat att tgg gat aac atg acc Ser Ser Trp Ser Asn Lys Ser Gln Glu Asp Ile Trp Asp Asn Met Thr 595 600 605	1824

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tgg atg cag tgg gat aga gaa att agt aat tac aca ggc aca ata tat Trp Met Gln Trp Asp Arg Glu Ile Ser Asn Tyr Thr Gly Thr Ile Tyr	610	615	620	1872	
agg tta ctt gaa gac tcg caa aac cag cag gag aaa aat gaa aaa gat Arg Leu Leu Glu Asp Ser Gln Asn Gln Glu Lys Asn Glu Lys Asp	625	630	635	640	1920
tta tta gca ttg gac agt tgg aaa aac ttg tgg aat tgg ttt aac ata Leu Leu Ala Leu Asp Ser Trp Lys Asn Leu Trp Asn Trp Phe Asn Ile	645	650	655	1968	
aca aat tgg ctg tgg tat ata aaa ata ttc atc atg ata gta gga ggc Thr Asn Trp Leu Trp Tyr Ile Lys Ile Phe Ile Met Ile Val Gly Gly	660	665	670	2016	
ttg ata ggt ttg aga ata att ttt ggt gta ctc gct ata gtg aaa aga Leu Ile Gly Leu Arg Ile Ile Phe Gly Val Leu Ala Ile Val Lys Arg	675	680	685	2064	
gtt agg cag gga tac tca cct ttg tcg ttt cag acc ctt acc cca agc Val Arg Gln Gly Tyr Ser Pro Leu Ser Phe Gln Thr Leu Thr Pro Ser	690	695	700	2112	
ccg agg ggt ccc gac agg ctc gga aga atc gaa gaa ggt gga gag Pro Arg Gly Pro Asp Arg Leu Gly Arg Ile Glu Glu Gly Gly Glu	705	710	715	720	2160
caa gac aaa gac aga tcc att cga tta gtg agc gga ttc tta gca ctt Gln Asp Lys Asp Arg Ser Ile Arg Leu Val Ser Gly Phe Leu Ala Leu	725	730	735	2208	
gcc tgg gac gat ctg cgg agc ctg tgc ctc ttc agc tac cac cac ttg Ala Trp Asp Asp Leu Arg Ser Leu Cys Leu Phe Ser Tyr His His Leu	740	745	750	2256	
aga gac ttc ata ttg att gca gcg aga gca gcg gaa ctt ctg gga cgc Arg Asp Phe Ile Leu Ile Ala Ala Arg Ala Ala Glu Leu Leu Gly Arg	755	760	765	2304	
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gga aat ctt gtg cag tat ggg ggt ctg gag cta aaa aga agt gct att Gly Asn Leu Val Gln Tyr Gly Gly Ile Glu Leu Lys Arg Ser Ala Ile	785	790	795	800	2400
aaa ctg ttt gat acc ata gca ata gca gta gct gaa gga aca gat agg Lys Leu Phe Asp Thr Ile Ala Ile Ala Val Ala Glu Gly Thr Asp Arg	805	810	815	2448	
att ctt gaa gta ata cag aga att tgt aga gct atc cgc cac ata cct Ile Leu Glu Val Ile Gln Arg Ile Cys Arg Ala Ile Arg His Ile Pro	820	825	830	2496	

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 835 840

<210> 19
 <211> 844
 <212> PRT
 <213> Artificial Sequence

<220>
 <223> Description of Artificial Sequence; Note =
 synthetic construct

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 Gly Thr Leu Gly Phe Trp Met Ile Ile Ile Cys Arg Val Val Gly Asn
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 Leu Asn Leu Trp Val Thr Val Tyr Tyr Gly Val Pro Val Trp Lys Glu
 35 40 45
 Ala Lys Thr Thr Leu Phe Cys Ala Ser Asp Ala Lys Ala Tyr Asp Lys
 50 55 60
 Glu Val His Asn Val Trp Ala Thr His Ala Cys Val Pro Thr Asp Pro
 65 70 75 80
 Asn Pro Arg Glu Ile Val Leu Glu Asn Val Thr Glu Asn Phe Asn Met
 85 90 95
 Trp Lys Asn Asp Met Val Asp Gln Met His Glu Asp Ile Ile Ser Leu
 100 105 110
 Trp Asp Gln Ser Leu Lys Pro Cys Val Lys Leu Thr Pro Leu Cys Val
 115 120 125
 Thr Leu Asn Cys Thr Asn Ala Pro Ala Tyr Asn Asn Ser Met His Gly
 130 135 140
 Glu Met Lys Asn Cys Ser Phe Asn Thr Thr Glu Ile Arg Asp Arg
 145 150 155 160
 Lys Gln Lys Ala Tyr Ala Leu Phe Tyr Lys Pro Asp Val Val Pro Leu
 165 170 175
 Asn Arg Arg Glu Glu Asn Asn Gly Thr Gly Glu Tyr Ile Leu Ile Asn
 180 185 190
 Cys Asn Ser Ser Thr Ile Thr Gln Ala Cys Pro Lys Val Thr Phe Asp
 195 200 205
 Pro Ile Pro Ile His Tyr Cys Ala Pro Ala Gly Tyr Ala Ile Leu Lys
 210 215 220
 Cys Asn Asn Lys Thr Phe Asn Gly Thr Gly Pro Cys Asn Asn Val Ser
 225 230 235 240
 Thr Val Gln Cys Thr His Gly Ile Met Pro Val Val Ser Thr Gln Leu
 245 250 255
 Leu Leu Asn Gly Ser Leu Ala Glu Glu Glu Ile Ile Ile Arg Ser Glu
 260 265 270
 Asn Leu Thr Asn Asn Ile Lys Thr Ile Ile Val His Leu Asn Lys Ser
 275 280 285
 Val Glu Ile Val Cys Thr Arg Pro Asn Asn Asn Thr Arg Lys Ser Ile
 290 295 300

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Arg Ile Gly Pro Gly Gln Thr Phe Tyr Ala Thr Gly Glu Ile Ile Gly
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 Asn Ile Arg Glu Ala His Cys Asn Ile Ser Lys Ser Asn Trp Thr Ser
 325 330 335
 Thr Leu Glu Gln Val Lys Lys Lys Leu Lys Glu His Tyr Asn Lys Thr
 340 345 350
 Ile Glu Phe Asn Pro Pro Ser Gly Gly Asp Leu Glu Val Thr Thr His
 355 360 365
 Ser Phe Asn Cys Arg Gly Glu Phe Phe Tyr Cys Asn Thr Thr Lys Leu
 370 375 380
 Phe Ser Asn Asn Ser Asp Ser Asn Asn Glu Thr Ile Thr Leu Pro Cys
 385 390 395 400
 Lys Ile Lys Gln Ile Ile Asn Met Trp Gln Lys Val Gly Arg Ala Met
 405 410 415
 Tyr Ala Pro Pro Ile Glu Gly Asn Ile Thr Cys Lys Ser Asn Ile Thr
 420 425 430
 Gly Leu Leu Leu Thr Arg Asp Gly Gly Lys Asn Thr Thr Asn Glu Ile
 435 440 445
 Phe Arg Pro Gly Gly Asn Met Lys Asp Asn Trp Arg Ser Glu Leu
 450 455 460
 Tyr Lys Tyr Lys Val Val Glu Ile Glu Pro Leu Gly Val Ala Pro Thr
 465 470 475 480
 Lys Ser Lys Arg Arg Val Val Glu Arg Glu Lys Arg Ala Val Gly Leu
 485 490 495
 Gly Ala Val Leu Leu Gly Phe Leu Gly Ala Ala Gly Ser Thr Met Gly
 500 505 510
 Ala Ala Ser Ile Thr Leu Thr Val Gln Ala Arg Gln Leu Leu Ser Gly
 515 520 525
 Ile Val Gln Gln Gln Ser Asn Leu Leu Arg Ala Ile Glu Ala Gln Gln
 530 535 540
 His Met Leu Gln Leu Thr Val Trp Gly Ile Lys Gln Leu Gln Thr Arg
 545 550 555 560
 Val Leu Ala Ile Glu Arg Tyr Leu Lys Asp Gln Gln Leu Leu Gly Leu
 565 570 575
 Trp Gly Cys Ser Gly Lys Ile Ile Cys Thr Thr Ala Val Pro Trp Asn
 580 585 590
 Ser Ser Trp Ser Asn Lys Ser Gln Glu Asp Ile Trp Asp Asn Met Thr
 595 600 605
 Trp Met Gln Trp Asp Arg Glu Ile Ser Asn Tyr Thr Gly Thr Ile Tyr
 610 615 620
 Arg Leu Leu Glu Asp Ser Gln Asn Gln Glu Lys Asn Glu Lys Asp
 625 630 635 640
 Leu Leu Ala Leu Asp Ser Trp Lys Asn Leu Trp Asn Trp Phe Asn Ile
 645 650 655
 Thr Asn Trp Leu Trp Tyr Ile Lys Ile Phe Ile Met Ile Val Gly Gly
 660 665 670
 Leu Ile Gly Leu Arg Ile Ile Phe Gly Val Leu Ala Ile Val Lys Arg
 675 680 685
 Val Arg Gln Gly Tyr Ser Pro Leu Ser Phe Gln Thr Leu Thr Pro Ser
 690 695 700
 Pro Arg Gly Pro Asp Arg Leu Gly Arg Ile Glu Glu Glu Gly Gly Glu
 705 710 715 720
 Gln Asp Lys Asp Arg Ser Ile Arg Leu Val Ser Gly Phe Leu Ala Leu
 725 730 735

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Ala Trp Asp Asp Leu Arg Ser Leu Cys Leu Phe Ser Tyr His His Leu
740 745 750
Arg Asp Phe Ile Leu Ile Ala Ala Arg Ala Ala Glu Leu Leu Gly Arg
755 760 765
Ser Ser Leu Arg Gly Leu Gln Arg Gly Trp Glu Ala Leu Lys Tyr Leu
770 775 780
Gly Asn Leu Val Gln Tyr Gly Gly Leu Glu Leu Lys Arg Ser Ala Ile
785 790 795 800
Lys Leu Phe Asp Thr Ile Ala Ile Ala Val Ala Glu Gly Thr Asp Arg
805 810 815
Ile Leu Glu Val Ile Gln Arg Ile Cys Arg Ala Ile Arg His Ile Pro
820 825 830
Ile Arg Ile Arg Gln Gly Phe Glu Ala Ala Leu Gln
835 840

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